

MECHANISMS UNDERLYING A UNIQUE FORM OF NEUROENDOCRINE ADAPTATION IN OSMOSENSITIVE SUPRAOPTIC NEURONS

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ABSTRACT

The neurohormonal mechanisms underlying the regulation of extracellular osmolality are of critical physiological importance. These mechanisms act to maintain the osmolality of human plasma close to a “set-point” of about 290 milliosmoles per litre. The magnocellular neurosecretory cells (MNCs) of the supraoptic nucleus (SON), synthesize and secrete the neurohypophysial hormones vasopressin (VP) and oxytocin (OT). The primary hormonal regulator of osmolality is VP, which is released by the MNCs as a function of plasma osmolality and acts by controlling water reabsorption at the kidneys. MNCs decrease their volume and thus plasma membrane tension in response to acute increases in external osmolality and lack the compensatory mechanisms that limit volume changes in most cell types. This enables them to transduce changes in osmolality into changes in excitability via a mechanosensitive cation channel. It has been shown *in vivo* that sustained increases in plasma osmolality, however, cause marked hypertrophy of the MNCs that is part of a structural and functional adaptation that is thought to enable the MNCs to secrete large quantities of VP for prolonged periods. The mechanism of this important structural and functional adaptation of MNCs is difficult to address in an *in vivo* preparation and so an *in vitro* model of acutely isolated MNCs was used to pharmacologically assess the hypertrophy. It was observed that MNCs exposed to sustained hypertonic solutions, underwent an immediate shrinkage followed by a hypertrophy over 90 minutes and quickly recovered when reintroduced to isotonic conditions. This effect was found to depend on the size of the increase in osmolality, as smaller increases in osmolality resulted in smaller shrinkage and hypertrophy of the MNCs. Hypertrophy was shown to be independent of cell volume regulatory processes as inhibitors of the $\text{Na}^+\text{-K}^+\text{-Cl}^-$ cotransporter did not affect hypertrophy. Hypertrophy was also shown to be dependent on activation of phospholipase C (PLC) and protein kinase C

(PKC), as adding inhibitors of these enzymes to the hypertonic solution prevented hypertrophy. Hypertrophy could occur in isotonic conditions by inducing cell depolarization, increasing intracellular calcium ($[Ca^{2+}]_i$) and by activating PKC, thus showing each of these processes are involved in hypertrophy. Recovery from hypertrophy depends upon dynamin-mediated endocytosis as blocking dynamin function prevented recovery. In addition, exposing the MNCs to hypotonic solution resulted in an immediate enlargement followed by a sustained decrease in cell size. Finally, exposing acutely isolated MNCs to hypertonic solution for two hours resulted in a 37% increase in the immunolabeling of the L-type Ca^{2+} channel $Ca_v1.2$ subunit. This increase in $Ca_v1.2$ immunolabeling does not depend on action potential firing as adding tetrodotoxin (TTX) to the hypertonic solution failed to prevent the increase. This project will help to elucidate the mechanisms underlying this interesting example of neuroendocrine adaptation and will help us to understand the regulation of body fluid balance during chronic challenges as seen in the elderly and chronically ill.

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DEDICATION

I would like to dedicate this thesis to my Family: Mom, Dad and Sister, as a small symbol of my gratitude for their unconditional love, insurmountable encouragement and unwavering support, which allowed me to overcome my biggest challenges and pursue my most passionate dreams.

TABLE OF CONTENTS

PERMISSION TO USE.....	i
ABSTRACT	ii
ACKNOWLEDGMENTS	iv
DEDICATION.....	vi
TABLE OF CONTENTS	vii
LIST OF FIGURES.....	x
LIST OF ABBREVIATIONS	xi
CHAPTER 1: INTRODUCTION	1
1.1. Osmolality and Osmoregulation	1
1.1.1. Central and Peripheral Osmoreceptors	3
1.1.2. Magnocellular Neurosecretory Cells (MNCs)	6
1.1.2.1. Morphology	7
1.1.2.2. Neurohypophysial Hormones	8
1.1.2.3. MNC Electrical Activity	9
1.2 Osmotic Adaptations	14
1.2.1 Short-term adaptations	14
1.2.1.1 Activation of PLC and changes in PIP ₂ levels.....	17
1.2.2 Long-term adaptations	19
1.2.2.1 Structural adaptations	20
1.2.2.2 Functional adaptations	21
1.2.2.3 Changes in cell surface levels of channels and receptors	21
1.2.2.5 Translocation of channels and receptors in other neurons.....	24
1.2.2.6 PKC involvement in translocation	25
1.3 Recent findings from the lab.....	27
CHAPTER 2: HYPOTHESIS AND OBJECTIVES	30
CHAPTER 3: MATERIALS AND METHODS.....	32
3.1 Chemicals	32
3.2 Animals and cell preparation	32

3.3 Morphometry experiments.....	33
3.4 Fluorescence images of hypertrophy	34
3.5 Immunocytochemistry of Cav1.2 subunit.....	34
3.6 Data analysis	35
CHAPTER 4: RESULTS.....	36
4.1 Osmotically evoked hypertrophy occurs at physiological osmolalities in a dose-dependent manner	36
4.2 Osmotically-evoked hypertrophy occurs independently of cell volume regulatory processes	40
4.3 Reversal of hypertrophy depends upon dynamin-mediated endocytosis	42
4.4 The activation of PLC and PKC are requirements for osmotically-evoked hypertrophy.....	44
4.5 Hypertrophy can be induced by activating PKC	46
4.6 Hypertrophy can be activated by depolarization of the MNCs	48
4.7 Hypertrophy can be induced by increasing $[Ca^{2+}]_i$	50
4.8 Exposure of MNCs to hypo-osmolar solutions results in a sustained decrease in soma size.....	52
4.9 Immunofluorescence of L-type Ca^{2+} subunit Cav1.2 in MNCs, increases after hypertonic exposure.....	54
CHAPTER 5: DISCUSSION.....	57
5.1 Osmotically-induced structural changes.....	57
5.1.1 Osmotically-evoked hypertrophy occurs <i>in vitro</i> and depends on specific cellular processes	58
5.1.1.1 Hypertrophy of MNCs is dose-dependent	61
5.1.1.2 Hypertrophy does not involve NKCC1-mediated cell volume regulation	61
5.1.1.3 Dynamin-dependent endocytosis governs the MNC recovery from hypertrophy	63
5.1.1.4 Activation of the PLC-PKC pathway is required for MNC hypertrophy	64
5.1.1.5 Cell depolarization and Ca^{2+} influx can cause MNC hypertrophy ..	65
5.1.1.6 Hypotonicity evokes reversible hypotrophy in MNCs	66
5.2 Osmotically-induced functional changes.....	67

5.2.1 Immunostaining of Cav1.2 L-type Ca ²⁺ channel subunit increases in MNCs exposed to sustained hypertonicity	67
5.3 Physiological consequences of MNC hypertrophy and increases in Cav1.2 subunit	68
5.4 Future directions	71
CHAPTER 6: CONCLUSION	75
CHAPTER 7: REFERENCES	76

LIST OF FIGURES

Figure 4.1: Fluorescence images of a MNC undergoing shrinkage and hypertrophy	38
Figure 4.2: Increases in osmolality evoke reversible hypertrophy in osmosensitive MNCs of the SON in a dose-dependent manner.....	39
Figure 4.3: Osmotically-evoked hypertrophy does not require activation of NKCC1	41
Figure 4.4: Reversal of osmotically-evoked hypertrophy depends on dynamin-dependent endocytosis.....	43
Figure 4.5: Osmotically-evoked hypertrophy involves activation of PLC and PKC	45
Figure 4.6: Hypertrophy can be induced by activation of PKC.....	47
Figure 4.7: Hypertrophy is activated by cell depolarization.....	49
Figure 4.8: Hypertrophy is activated by influx of Ca^{2+}	51
Figure 4.9: Hypo-osmolality evokes a reversible decrease in MNC size	53
Figure 4.10: Pictures of MNCs showing increased immunofluorescence of the $\text{Ca}_v1.2$ subunit after hypertonic exposure.....	55
Figure 4.11: Bar graph of MNCs showing increased immunofluorescence of the $\text{Ca}_v1.2$ subunit after hypertonic exposure.....	56

LIST OF ABBREVIATIONS

AHP	Afterhyperpolarizing afterpotential
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
AQP	Aquaporin
ATP	Adenosine triphosphate
BAPTA-AM	1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis (acetoxymethyl ester)
[Ca²⁺]_i	Intracellular Ca ²⁺ concentration
cAMP	3'-5'-cyclic adenosine monophosphate
ClC	Chloride channel
CREB	cAMP response element-binding protein
CSA	Cross-sectional area
DAG	Diacyl-glycerol
DAP	Depolarizing afterpotential
DIC	Differential interference contrast
ECF	Extracellular fluid
EGTA	Ethylene glycol tetraacetic acid
ER	Endoplasmic reticulum
GABA	γ -Aminobutyric acid
GluR	AMPA subunit
GPCR	G protein-coupled receptors
GTP	Guanosine-5'-triphosphate

HAP	Hyperpolarizing afterpotential
Hz	Hertz
ICF	Intracellular fluid
IP₃	Inositol 1,4,5-trisphosphate
LTD	Long-term depression
LTP	Long-term potentiation
MNC	Magnocellular neurosecretory cell
MnPO	Median preoptic nucleus
mosmol kg⁻¹	Milliosmoles per kilogram of solvent
mosmol L⁻¹	Milliosmoles per liter of solvent
NKCC	Na ⁺ -K ⁺ -Cl ⁻ cotransporter
NMDAR	N-methyl-D-aspartate receptor
NSF	<i>N</i> -ethylmaleimide sensitive fusion protein
OVL	Organum vasculosum of the lamina terminalis
PBS	Phosphate-buffered saline
PGC	PIPES-glucose-calcium buffer
PIP₂	Phosphatidylinositol 4,5-bisphosphate
PIPES	Piperazine-N,N'-bis(2-ethanesulfonic acid)
PKC	Protein kinase C
PLC	Phospholipase C
PMA	Phorbol-12-myristate-13-acetate
PVN	Paraventricular nucleus
RVD	Regulated volume decrease

RVI	Regulated volume increase
SEM	Standard error of mean
SER	Smooth endoplasmic reticulum
SFO	Subfornical organ
SIC	Stretch-inactivated cation channel
SNARE	Soluble NSF attachment protein receptor
SON	Supraoptic nucleus
TRP	Transient receptor potential channel
TTX	Tetrodotoxin
VAMP	Vesicle-associated membrane protein
VGCC	Voltage-gated Ca ²⁺ channel
VP	Vasopressin
κOR	κ-opioid receptor

CHAPTER 1: INTRODUCTION

1.1. Osmolality and Osmoregulation

The colligative properties of solutions are important elements of their physical properties and depend upon the amount of solute in a given amount of solvent (Pinarbasi *et al.*, 2009). Among the colligative properties, osmotic pressure remains the most essential in cellular physiology (Sanger *et al.*, 2001). In multicellular organisms, the electrolyte and organic molecule exchange occurs between the intracellular fluid (ICF) and extracellular fluid (ECF) (Fleischhauer *et al.*, 1995). The ICF comprises of the cell's cytosol (Luby-Phelps, 1999) while the ECF consists of the interstitial fluid (between cells) and the blood plasma (Gauer *et al.*, 1970). Electrolyte balance is imperative to maintain fluid balance within an organism (Rolls & Phillips, 1990) and the processes for maintenance occur for each body fluid compartment (Sawka & Montain, 2000). These processes include organ-level regulation by the renal system (filtration and reabsorption) (Bankir *et al.*, 1989) and cellular-level regulation by osmosis, the flow of water from a region of higher concentration to lower concentration through passive diffusion (Metheny, 2011). At the level of cells, the plasma membrane is an important organelle that partitions the ECF from the ICF and allows water to travel across its membrane through specialized water pores (Curran & Solomon, 1957). The osmolality indicates the moles of osmolytes (solutes such as proteins, small molecules and ions) in a given kilogram of solvent (mosmol kg^{-1}) (Bourque *et al.*, 2007). Osmolarity is an analogous measure, which indicates the moles of osmolytes in a given liter of solvent (mosmol L^{-1}) (Erstad, 2003).

Mammals are termed osmoregulators, which means that they maintain ECF osmolality near a set-point (about 300 mosmol kg⁻¹ in mammals) (Bourque, 2008). An ECF osmolality lower than normal is termed hypotonic while an osmolality higher is termed hypertonic. When the ECF undergoes osmotic changes, water flows through the cell membrane by means of osmosis to balance the ECF and ICF osmolality (Halperin & Skorecki, 1986; Bourque, 2008). This tendency to draw water between compartments is termed osmotic pressure and the difference in osmolalities between the two compartments is the driving force (Guyton & Hall, 2006; Boron & Boulpaep, 2008). The most important cation in the ECF is sodium (Na⁺) (Terry, 1994) and the main regulatory processes that occur to maintain ECF osmolality do so by regulating Na⁺ and water (Guyton & Hall, 2006; Bourque, 2008). The regulation of Na⁺ levels can occur through changes in Na⁺ appetite (Weisinger *et al.*, 1983; Blackburn *et al.*, 1993) or through modification of Na⁺ excretion (natriuresis) (Huang *et al.*, 1996; Andersen *et al.*, 2002). Water levels can be altered through urine output (Verney, 1947; Bourque, 2008), thirst (Zerbe & Robertson, 1983; Baylis, 1987; Egan *et al.*, 2003; McKinley & Johnson, 2004), sweating (Fortney *et al.*, 1984; Saat *et al.*, 2005; Bourque, 2008) and evaporation (Tucker, 1968). In mammals, homeostatic mechanisms employed during hyperosmotic challenges (dehydration or high salt consumption) include increased release of vasopressin (VP), a hormone that suppresses diuresis via increased renal water reuptake (Adolph & Ericson, 1927; De Wardener & Del Greco, 1955; Orloff *et al.*, 1958; Guyton & Hall, 2006; Boron & Boulpaep, 2008) and motivation of water drinking (Thornton, 1986; Schoorlemmer *et al.*, 2000). During conditions when the ECF is hypo-osmolar, the release of VP is reduced and subsequently the renal water reuptake is reduced, increasing the urine output (Guyton & Hall, 2006; Bourque, 2008).

The significance of maintaining a physiological osmolality of the ECF can be evident during the acute changes that occur at the cellular level (Bourque & Oliet, 1997; Boron & Boulpaep, 2008; Bourque, 2008). When the ECF is hypo-osmolar, water will be drawn into cells and cause cell swelling (Lang *et al.*, 1998b; Lang, 2007; Boron & Boulpaep, 2008). During hyperosmolar ECF conditions, water is drawn out of the cell causing it to shrink (Lang *et al.*, 1998b; Lang, 2007; Boron & Boulpaep, 2008). The flow of water in and out of the cell is through water channels and is dictated by osmosis until the ICF and ECF equalize in osmolality (Lang *et al.*, 1998b; Lang, 2007; Boron & Boulpaep, 2008). As an adaptive process, many cells can undergo compensatory processes to counteract shrinkage and enlargement to maintain cell volume, known as regulatory volume increase (RVI) and regulatory volume decrease (RVD), respectively (Bourque & Oliet, 1997; Lang *et al.*, 1998a). The mechanisms employed against cell volume changes act by regulating internal osmolytes by transporting them across the cell membrane so that the osmolality of the ECF and ICF come to a balance (Lang *et al.*, 1998a; Boron & Boulpaep, 2008). The maintenance of the ECF osmolality is crucial for the central nervous system, especially the brain, as extreme changes in volume can damage the tissue encased within the inflexible cranium (Bourque, 2008). When the osmolality changes, severe structural and functional damage can result leading to hyperexcitability and neuronal death (Bourque, 2008).

1.1.1. Central and Peripheral Osmoreceptors

Some aquatic animals have specific evolutionary adaptations that allow them to withstand changes to osmolality through optimization of cell volume regulatory mechanisms (Bourque, 2008; Aladin & Plotnikov, 2009). These animals are termed osmoconformers because the osmolality of their cells changes with the osmolality of their environment (Bourque, 2008). Osmoregulators, however, have specific homeostatic mechanisms that govern the ECF osmolality near a “set-point”

(Bourque, 2008). This demonstrates that there are specific sensors in the organism that can detect changes in the ECF osmolality and subsequently govern the compensatory processes to attain osmotic homeostasis (Bourque & Oliet, 1997; Bourque, 1999; Bourque *et al.*, 2007; Bourque, 2008).

It has been shown that infusions of hypertonic solutions cause cellular dehydration, and that this is necessary to induce VP release and stimulate the thirst response (Gilman, 1937; Verney, 1947). Later studies documented the existence of specific osmoreceptor cells that can detect the ECF osmolality changes and showed that these cells reside within the brain (Jewell & Verney, 1957). Based on the fact that the cells are neuronal cells that can detect changes in ECF osmolality, researchers concluded that it is the cell's resting electrical activity that defines the set-point of ECF osmolality (Bourque, 2008). The cell's resting membrane potential can be governed by membrane stretching, which can be transduced into changes in ion channel activity and membrane potential (Verney, 1947; Bourque, 2008). Thus changes in osmolality result in changes in the tendency to fire action potentials (Bourque, 2008). Early studies documented the effects of inducing hypertonicity in blood plasma as an effective stimulator for thirst (Bourque *et al.*, 1994). Other studies have shown that the thirst response is dependent upon changes in cell volume (water efflux from cytoplasm), as permeable osmolytes are not able to induce the thirst response (Gilman, 1937). This was an instrumental finding suggesting the individual cell size changes can induce the osmoregulatory behaviour. It was not certain, however if this was the collective response from all cells in the animal or if there were cells that were specific for sensing changes in osmolality (Bourque *et al.*, 1994; Bourque, 2008).

One of the first studies attempting to identify the location of central nervous system osmoreceptors showed that VP could be released by increasing the carotid artery blood osmolality by 1% (Verney,

1947). Using surgical methods to isolate arterial blood supplies, they found that the site for osmotic receptors were found in the ventral area of the hypothalamus (Verney, 1947). This specific site within the hypothalamus contains the supraoptic and paraventricular nuclei (SON and PVN), which have the magnocellular neurosecretory cells (MNCs) (Verney, 1947; Bourque & Oliet, 1997; Bourque, 2008). As an extension to the findings it was found that osmolytes impermeable to the blood-brain barrier do not affect urine output (diuresis) suggesting that the central sites of osmoreception must be located in areas that do not have a blood-brain barrier (Thrasher *et al.*, 1980). This finding led to the investigation of numerous sites within the neural circumventricular organs such as the organum vasculosum of the lamina terminalis (OVLT) and the subfornical organ (SFO) and that lesions in these sites impair neurohormonal regulation of plasma osmolality (Thrasher *et al.*, 1980). It has been shown that the OVLT and SFO innervate the MNCs through the median preoptic nucleus (MnPO) and that the electrical and hypertonic stimulation of these upstream areas can cause excitation in the MNCs and the release of VP and oxytocin (OT) (Bourque *et al.*, 1994).

This neural circuitry is known to underlie the central nervous system control of osmoregulation. Osmoregulatory behaviours such as thirst can take time to cause a change in plasma osmolality but thirst can be quenched and osmoregulatory hormonal influences can change much before (Bourque *et al.*, 1994). This observation suggests the possibility of peripheral osmoreceptors that can detect changes peripherally before a systemic change in osmolality can be detected by central osmoreceptors (Bourque *et al.*, 1994). It is known that absorption of osmolytes and fluids is through the splanchnic mesentery artery, hepatic portal vein, liver and finally to the systemic circulation (Boron & Boulpaep, 2008). This finding led to the possibility that the peripheral osmoreceptors may be present somewhere in these sites (Bourque *et al.*, 1994). It has been shown

that infusing water or hypertonic saline into the hepatic portal vein, causes increases and decreases in diuresis respectively and that this can change the electrical activity of MNCs and the subsequent release of VP (Haberich, 1968). Electrophysiological studies have confirmed that the hepatic branch of the vagus nerve has modulated firing patterns after changes in the osmolality of the hepatic portal circulation (Adachi *et al.*, 1976). The location of these peripheral receptors makes them very important in anticipating the osmotic effects of the nutrients and fluids absorbed allowing the osmoregulatory processes to buffer these changes (Bourque *et al.*, 1994). Further, it has been shown in mice that afferent projections in the thoracic dorsal root ganglion have innervations and nerve endings in the hepatic portal system (Choi-Kwon & Baertschi, 1991). This confirms findings showing functional osmosensation in the peripheral hepatic circulation. The transduction mechanism to sense changes in osmolality are electrical and are dependent upon an osmotically sensitive ion channel, the transient receptor potential vanilloid 4 (TRPV4) channel as peripheral osmoreceptors of TRPV4 knockout mice were no longer activated with osmolality changes (Lechner *et al.*, 2011). Other peripheral osmoreceptors sites include within the gastrointestinal tract (Dooley & Valenzuela, 1984; Carlson *et al.*, 1997) and the oropharyngeal cavity (Kuramochi & Kobayashi, 2000).

1.1.2. Magnocellular Neurosecretory Cells (MNCs)

The MNCs are central osmoreceptors that synthesize and secrete the hormones VP and (OT) into the general circulation (Swaab *et al.*, 1975). The MNCs are located in the hypothalamic regions of the SON and PVN with an total abundance of about 10,000 in rats (Rhodes *et al.*, 1981) and 100,000 in humans (Manaye *et al.*, 2005). The SON primarily contains MNCs with astrocytes (Tweedle & Hatton, 1977; Theodosis & Poulain, 1984) and few interneurons (Hatton, 1990; Armstrong, 1995) between the MNCs. MNCs synthesize either OT or VP (Sofroniew, 1982), and

send a single axon to the posterior pituitary gland through the median eminence (Brownstein *et al.*, 1980). This bundle of OT and VP MNC axons is known as the hypothalamic-neurohypophyseal tract. It is at the posterior pituitary that they secrete their hormones into the rich blood plexus (Brownstein *et al.*, 1980). Each axon ramifies into 2,000 – 10,000 terminals, which are dense in OT or VP containing vesicles (Brownstein *et al.*, 1980; Meeker *et al.*, 1991). The MNCs possess between one to three dendrites (Stern & Armstrong, 1998), most of which project to the ventral glial lamina (Armstrong & McNeill, 1982). The release of OT and VP can also occur through the dendrites as the presence of neurosecretory vesicles have been detected there (Ludwig, 1998b). In addition to secretion of neurohypophyseal hormones from the axon terminals studies have shown release from the somatodendritic regions, which is thought to have an important role in regulating function of MNCs (Ludwig, 1998b). The release of these neuropeptides is shown to have an autocrine and paracrine role as MNCs express the receptors for the neuropeptides and their activation leads to an increase in intracellular calcium $[Ca^{2+}]_i$ (Lambert *et al.*, 1994; Dayanithi *et al.*, 1996). Other somatodendritic secretions by MNCs include the opioid dynorphin (Whitnall *et al.*, 1983) and nitric oxide (through nitric oxide synthase) (Kadowaki *et al.*, 1994). Dynorphin has a role in terminating phasic firing (Brown & Bourque, 2004) and diminishes VP release (Brown *et al.*, 1999). The roles of nitric oxide in MNCs may include modulation of electrical firing patterns (Liu *et al.*, 1997) and an action on afferent neurons to modulate presynaptic transmission (Cui *et al.*, 1994).

1.1.2.1. Morphology

MNCs can be isolated and identified using methods that have been well established (Oliet & Bourque, 1992; Armstrong, 1995). As the name suggests, MNCs are large and 96% of cells isolated from the SON region that have a cross sectional area more than $160 \mu m^2$ are MNCs (Oliet

& Bourque, 1992). The commonly used protocols for *in vitro* enzymatic separation of MNCs from a single block of SON tissue has been shown to yield about 2-10% of the total cell number (100 – 500 MNCs) from rats (Oliet & Bourque, 1992).

1.1.2.2. Neurohypophysial Hormones

The primary role of MNCs is to synthesize and secrete OT and VP, which are hormones serving important roles in mammalian physiology and homeostasis (Swaab *et al.*, 1975; Bourque, 2008). The hormone VP plays a prominent role in osmoregulation by modulating urine output through its actions on the kidney collecting ducts (Guyton & Hall, 2006; Boron & Boulpaep, 2008). A minor role of VP is thermoregulation by modulating renal water uptake (Sharif-Naeini *et al.*, 2008). VP is also a vasoconstrictor that increases blood pressure by increasing arterial resistance (Toba *et al.*, 1991; Bourque, 2008). VP also has effects on behaviour including regulating thirst (Thompson *et al.*, 1987) and when released in the central nervous system it has roles in memory consolidation (Gash & Thomas, 1983; Gispen, 2011) and sexual mood (Murphy *et al.*, 1987). VP performs its physiological roles by acting on V_{1a}, V_{1b}, and V₂ G-protein coupled receptors (GPCRs) (Ball, 2007). The V_{1a} receptor is expressed on MNC plasma membranes and its activation causes autocrine inhibition of VP release. Antagonism of V_{1a} receptors switches the MNCs from silent to phasic firing (Ludwig & Leng, 1997; Brown *et al.*, 2004). Aquaporins are a class of carrier protein that facilitate water transport governed by osmotic forces (Agre, 2006). In the kidneys of mammals, aquaporin-2 (AQP2), aquaporin-3 (AQP3) and aquaporin-4 (AQP4) are specifically implicated in the water reabsorption process (Ball, 2007). At the cellular level of water homeostasis, VP acts on the V₂ receptor in the kidney and causes cyclic adenosine monophosphate (cAMP)-dependent translocation of AQP2 to the apical membrane of the collecting duct, via exocytotic fusion of vesicles containing AQP2 (Agre, 2006; Petersen, 2006; Ball, 2007). The

basolateral membrane contains AQP3 and AQP4, which allow water in the cell to depart to the surrounding ECF (Agre, 2006; Petersen, 2006; Ball, 2007). During normal conditions, the blood VP concentration is about 1-3 pg/mL (Forsling *et al.*, 1998) and the maintenance of that range induces renal water reabsorption of about 30 liters a day (Forsling *et al.*, 1998).

OT is a hormone that is involved mainly in female physiology of mammals, including lactation and the milk letdown reflex (Moon & Turner, 1959; Guyton & Hall, 2006; Boron & Boulpaep, 2008). OT is also known for its physiological role in inducing uterine contractions during labour (Takayanagi *et al.*, 2005; Boron & Boulpaep, 2008). OT is also involved in modulating behaviour including sexual arousal in men (Murphy *et al.*, 1987) and women (Blaicher *et al.*, 1999), and has a calming effect (high trust, low fear) (Kirsch *et al.*, 2005; Theodoridou *et al.*, 2009; Cardoso *et al.*, 2013; Lane *et al.*, 2013). In males, OT is known to be involved in contraction for sperm motility in the epididymis (Studdard *et al.*, 2002; Gupta *et al.*, 2008). The normal amounts of circulating OT is similar to VP, about 1-3 pg/mL (Forsling *et al.*, 1998). Physiological levels of OT is shown to have a natriuretic role in rats (Verbalis *et al.*, 1991). OT and VP are produced and packaged into secretory vesicles in the MNC cell bodies, then subsequently carried to the axon terminals of MNCs in the posterior pituitary (Theodosios *et al.*, 1978; Bicknell, 1988). During release of the hormones, the action potentials generated cause activation of voltage-gated Ca^{2+} channels (VGCCs), which allows Ca^{2+} influx, exocytotic fusion of the neuropeptide vesicles and release into the plexus of blood vessels in the posterior pituitary (Hatton, 1990; Armstrong, 1995).

1.1.2.3. MNC Electrical Activity

MNCs are neuronal cells that can detect changes in ECF osmolality (Bourque *et al.*, 2007; Bourque, 2008). Thus changes in osmolality result in changes in the membrane potential and the

tendency to fire action potentials (Oliet & Bourque, 1992; Bourque *et al.*, 2007; Bourque, 2008; Sharif-Naeini *et al.*, 2008) and hormone output (Bicknell, 1988). Under isotonic ECF conditions, the action potential firing is about 1-3 Hz, which is considered small and arrhythmic (Poulain & Wakerley, 1982; Bourque & Oliet, 1997). The activity pattern of MNCs changes during increases of as little as 3 mosmol/kg to more continuous and rapid firing (Poulain & Wakerley, 1982; Bourque & Oliet, 1997). The third pattern, phasic firing, is the most effectual for rapid secretion of VP from the axon terminals (Leng *et al.*, 1999; Brown *et al.*, 2013). Increases in osmolality also increase firing in OT MNCs (Brimble & Dyball, 1977; Brimble *et al.*, 1978), and this may be important in osmoregulation due to the effect of OT on natriuresis (Verbalis *et al.*, 1991). In phasic firing, MNCs fire for about 15 seconds followed by silent periods of about 10 seconds. At the beginning of each burst, MNCs display rapid firing (15-25 Hz) followed by slower firing (about 6 Hz) lasting till the end of the burst (Poulain & Wakerley, 1982; Brown, 2004; Brown & Bourque, 2006; Brown *et al.*, 2013). Phasic firing is the most efficient pattern for evoking hormone release because during continuous firing (>10 seconds) the release fatigues, which then recovers after a rest period (Bicknell *et al.*, 1984; Brown *et al.*, 2013). The firing patterns of MNCs are due to various intrinsic and extrinsic modulators (Brown *et al.*, 2013). Intrinsic factors include specific currents based on osmolality (stretch-inactivated cation channels; SICs) and activity-dependence (afterpotentials) (Oliet & Bourque, 1993a; Oliet & Bourque, 1993b).

The ability of MNCs to transduce changes in osmolality into changes in electrical potential is a property that is mediated through specific SICs (Oliet & Bourque, 1993b). On a physical level, osmolality changes cause cell shrinkage or enlargement during hypertonic and hypotonic conditions and it is these changes in cell size that are key to electrophysiological changes (Bourque, 2008). The SICs are plasma membrane channels that respond to membrane tension and so applying

positive pressure in a patch clamp setup mimics cell swelling and results in closing of the SICs causing hyperpolarization, lower action potential firing and reduced hormone release (Oliet & Bourque, 1993b; Bourque, 2008). In hypertonic conditions during MNC shrinkage, there is lower plasma membrane tension and so the SICs open and allow cation influx, increased action potential generation and increased hormone output (Oliet & Bourque, 1993b; Bourque, 2008). The mechanosensitivity of MNCs involves actin filaments as actin polymerization affects the mechanosensitivity of MNCs (Zhang *et al.*, 2007b; Bourque, 2008). The SIC is an ion channel that is part of the TRPV family. It is thought to be a specific N-terminal splice variant of TRPV1 (Sharif-Naeini *et al.*, 2008). The inability of TRPV1 knockout mice MNCs to be osmosensitive (i.e. to display osmolality-dependent cell activity) and inability to release normal amounts of VP, demonstrates the important role of SICs in transducing osmolality information into electrical signals (Sharif-Naeini *et al.*, 2008).

A recently identified osmosensitive current, the OKC, is a voltage-gated K^+ channel that may be important in regulating the firing patterns seen in MNCs (Zhang *et al.*, 2009). The role of the OKC is not completely clear as an osmolality-dependent outward K^+ current would be expected to hyperpolarize the cell rather than induce cell firing (Zhang *et al.*, 2009). An alternate hypothesis is that the OKC might be involved in maintaining the silent phase during phasic firing or at least slow the phasic firing activity to reach the silent phase (Zhang *et al.*, 2009).

The phasic burst firing pattern is very important in maximal hormonal release (Leng *et al.*, 1999) and their generation depends on both glutamatergic excitatory postsynaptic potentials and depolarizing afterpotentials (DAP) (Andrew & Dudek, 1983; Israel *et al.*, 2010). These DAPs are dependent on activity and are regulated by the membrane's intrinsic properties (Andrew & Dudek, 1983). $[Ca^{2+}]_i$ plays an important role in phasic firing by activating DAPs (Li *et al.*, 1995). MNCs

also have intrinsic currents that hyperpolarize the cell (inhibitory currents) including hyperpolarizing afterpotentials (HAP) and afterhyperpolarizing afterpotentials (AHP) (Armstrong *et al.*, 1994). These inhibitory currents are modulated by Ca^{2+} -dependent potassium (K^+) channels (Andrew & Dudek, 1983; Armstrong *et al.*, 1994). MNCs have silent phases and the mechanism of the termination of the bursts and prolonged silence are not completely understood (Andrew & Dudek, 1983; Roper *et al.*, 2004; Brown *et al.*, 2006), however the HAP and AHP could be involved in burst termination (Brown *et al.*, 2013). In addition, it was observed that dendritic release of dynorphin and activation of κ -opioid receptors, terminates phasic firing by decreasing the plateau potential and the probability of further spikes (Brown *et al.*, 2006). Thus the depolarizing DAP and hyperpolarizing HAP and AHP currents are involved in modulating MNC electrical activity in an activity-dependent manner to ensure maximal and efficient release of hormones. Extrinsic signaling from other neuronal populations, such as those in the OVLT, also serves to modulate MNC electrical activity and is a requirement for phasic firing patterns (Yang *et al.*, 1994; Richard & Bourque, 1995). The intrinsic and extrinsic activity modulations serve to regulate action potential firing, which is required for VGCC opening and hormonal output into the general circulation (Bourque *et al.*, 1994; Bourque & Olier, 1997; Bourque, 2008).

Ca^{2+} is a very important ion in cellular physiology and specific mechanisms are in place to maintain Ca^{2+} homeostasis (Carafoli, 1987). A very important role of Ca^{2+} is in second messenger systems that are involved in maintaining cellular functions during physiological and pathophysiological states (Petersen *et al.*, 2005). Ca^{2+} is buffered inside the cell within the endoplasmic reticulum (ER) (Koch, 1990) and is kept at intracellular concentrations of about 100 nM, a 10^6 dilution from the extracellular concentrations of 1-2 mM (Clapham, 2007). The mitochondrion requires Ca^{2+} for its oxidative phosphorylation activity and can be another buffering compartment for Ca^{2+}

(Zamponi, 2005). The reason that $[Ca^{2+}]_i$ is kept so low is because Ca^{2+} is involved in so many intracellular processes (Berridge *et al.*, 2003). When Ca^{2+} is required, it may rise to 400 nM or more (Wong *et al.*, 1991). In vertebrates, Ca^{2+} is important in bone as the phosphate and sulfate salt and it presents a very important reservoir for ECF Ca^{2+} when required (Ham, 1932; Boron & Boulpaep, 2008). Ca^{2+} has many prominent roles in various cell types such as neurotransmission in neurons (Ross, 1989), contraction in myocytes (Cheung *et al.*, 1982), vasodilation in endothelial cells (Cauvin *et al.*, 1983), and activation of the T-cell response in the immune system (Birx *et al.*, 1984). The entry of Ca^{2+} is through numerous membrane receptors such as TRP channels (Birnbaumer *et al.*, 1996), N-methyl D-aspartate receptors (NMDARs) (Bauer *et al.*, 2002) and VGCCs (Catterall *et al.*, 2005). VGCCs are important ports of Ca^{2+} entry that open in response to a depolarizing cell voltage (Catterall *et al.*, 2005). The VGCCs are very selective for Ca^{2+} (Catterall *et al.*, 2005). Each channels consists of an α_1 -subunit along with a γ -subunit, a β -subunit (except T-type) and a α_2 - δ complex (Catterall *et al.*, 2005). The α_1 -subunit is important for voltage sensing and for the Ca^{2+} selective pore (Catterall *et al.*, 2005). Different types of VGCCs exist including L-, N-, P-, Q-, R- and T-types which are well documented through electrophysiological, biophysical and pharmacological studies (Goldin *et al.*, 2000; Catterall *et al.*, 2005). The VGCCs are expressed in MNCs soma (T-, L-, R-, P- and N-) and nerve terminals (L-, N-, and Q-) (Fisher & Bourque, 1996; Fisher *et al.*, 2000).

Of particular importance to neuronal populations is the L-type Ca^{2+} channel, which is part of the Cav1 family (Goldin *et al.*, 2000; Catterall *et al.*, 2005). They play roles in neuronal plasticity (Galewski *et al.*, 1992; Hendricson *et al.*, 2003; Fossat *et al.*, 2007), neurotransmitter secretion (Perney *et al.*, 1986; Westenbroek *et al.*, 1990; Catterall, 1998) and development (Lipscombe *et al.*, 2004). Two main subtypes of L-type Ca^{2+} channels, Cav1.2 and Cav1.3, have been shown

through gene expression studies (Glasgow *et al.*, 2000) and immunohistochemistry (Joux *et al.*, 2001) to be present in MNC soma and axon terminals. Together, the L-type Ca^{2+} channels are responsible for about 25% of all the VGCC currents (Fisher & Bourque, 1995, 1996; Foehring & Armstrong, 1996). In other cell types, they are implicated in gene expression (Chung & Jan, 2006) and cell division (Loechner *et al.*, 2010). The gene expression regulatory control by $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ is due to the C-terminus of both channel subtypes, which allow them to interact with cAMP response element binding (CREB) protein (Burbach *et al.*, 2001). The C-terminus of $\text{Ca}_v1.2$ can act as a transcriptional regulator when transported to the nucleus (Burbach *et al.*, 2001). Pharmacological blockers of L-type Ca^{2+} channels include dihydropyridines and phenylalkylamines (Schuster *et al.*, 1996; Lipkind & Fozzard, 2003).

1.2 Osmotic Adaptations

With the evolution of the nervous system and in vertebrates with a stiff cranium, neuronal physiological processes are very much reliant on the chemical and physical environment (Bourque, 2008). Osmoregulators maintain ECF osmolality by active regulation of ionic concentrations to achieve isotonic ECF conditions and by specialized neuronal homeostatic control (Lang *et al.*, 1998a; Bourque, 2008). This section will discuss some of the structural and functional adaptations in both short and long term exposure to osmotic changes.

1.2.1 Short-term adaptations

MNCs are able to detect and modulate their physiological activity and hormonal output during short term changes in osmolality (Bourque *et al.*, 1994; Zhang & Bourque, 2003; Brown & Bourque, 2006; Bourque, 2008). The MNCs use SICs to transduce the change in osmolality to a change in electrical activity (Oliet & Bourque, 1993b; Sharif-Naeini *et al.*, 2008). All cells,

including MNCs, shrink immediately following exposure to hypertonic solutions and this shrinkage relieves membrane tension and subsequently opens SICs (Oliet & Bourque, 1993b; Zhang & Bourque, 2003). This opening of the SICs allows influx of cations, depolarization, and action potential firing (Bourque, 2008). During hypo-osmotic exposure, the cells swell, increasing membrane tension and closing the SICs, resulting in hyperpolarization and decreased MNC cell firing (Bourque, 2008).

Animals have evolved cellular pathways to counteract the acute volume changes; these are termed regulated volume increase (RVI) and regulated volume decrease (RVD) (Lang *et al.*, 1998a; Lang, 2007). During the initial phase of a cell in a hypertonic solution, osmosis dictates that water will rush out of the cell due to the newly established osmotic gradient and cause overall cell shrinkage (Lang *et al.*, 1998b; Lang, 2007). As outlined, the cell will undergo RVI, which occurs primarily by ion influx to re-establish the inner osmotic strength which preserves the remaining water (Lang *et al.*, 1998a; Lang, 2007). The shrinkage of the cell leads to the activation of specific channels and cotransporters involved in cell volume regulation, such as the $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter, Na^+/H^+ exchanger and the $\text{Cl}^-/\text{HCO}_3^-$ exchanger (Lang *et al.*, 1998b). Both the Na^+/H^+ exchanger and $\text{Cl}^-/\text{HCO}_3^-$ exchanger act to replenish intracellular NaCl, while the excess Na^+ (through the $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter or Na^+/H^+ exchanger) is extruded by a Na^+/K^+ ATPase leading to a net influx of KCl (Lang *et al.*, 1998b; Gamba, 2005). Some Na^+ channels may activate and the depolarization causes Cl^- influx. (Lang *et al.*, 1998b; Wehner *et al.*, 2003).

When cells are exposed to hypo-osmolar conditions, osmosis determines that water will rush in to equilibrate the ICF and ECF osmolality causing cellular swelling (Bourque, 2008). To protect from further expansion, the cells recruit RVD processes, which act by extruding K^+ and other anions (Okada, 2006; Wehner, 2006). The main K^+ channels involved in its efflux are $\text{K}_v1.3$ and $\text{K}_v1.5$

and anion channels include the chloride channels ClC-2 and ClC-3 (Lang *et al.*, 1998b). The increased membrane tension may also activate stretch-activated cation channels, which may indirectly lead to K⁺ efflux by Ca²⁺ entry and subsequent activation of Ca²⁺-sensitive K⁺ channels (Lang *et al.*, 1998b; Lang, 2007). In human salivary gland cells, swelling results in release of adenosine triphosphate (ATP), which acts in a autocrine fashion on purinergic receptors (P2Y₂) causing Ca²⁺ release and opening of K⁺ channels (Ryu *et al.*, 2010).

In addition to cell volume regulation, the electrophysiological properties of MNCs also change during short term osmotic stress. The cell capacitance represents the charge separation capacity of a cell and this is dependent upon the cellular membrane surface area (Hille, 2001; Partridge & Partridge, 2003; Boron & Boulpaep, 2008; Bourque, 2008). This electrophysiological parameter can be monitored and can give important information on cellular morphometry and the dynamics of cellular membranes during the course of a hypertonic or hypotonic shock (Boron & Boulpaep, 2008; Bell & Rhoades, 2009). Exposure to hypotonic and hypertonic solutions causes a respective decrease and increase in MNC conductance (Bourque & Oliet, 1997). Changes in cell volume and conductance occur on similar time frames and are both reversible (Bourque & Oliet, 1997). The effect of short term osmotic changes of MNCs on morphometry and capacitance are different than those of non-osmosensitive neurons such as hippocampal neurons (Zhang & Bourque, 2003). When exposed to hypotonic or hypertonic solutions, MNCs and hippocampal neurons displayed the normal cellular swelling or shrinkage, respectively (Zhang & Bourque, 2003; Bourque, 2008). It was noted however that over time, during slow osmotic ramps the hippocampal neuron volume did not fluctuate significantly, indicating that RVI and RVD operate in non-MNC neurons (Zhang & Bourque, 2003). In experiments with a fast step of hypotonic exposure, both MNCs and hippocampal neurons showed rapid swelling, which was maintained in MNCs, but hippocampal

neurons showed recovery starting about 100 seconds after exposure, further indicating the rapid RVD seen in these cell types (Zhang & Bourque, 2003). In the reverse condition of a fast hypertonic exposure, both MNCs and hippocampal neurons shrunk but the hippocampal neurons shrunk less, indicating a dynamic and fractional RVI (Zhang & Bourque, 2003). These data suggest that MNCs lack the cell volume regulatory processes which protect most other cell types from swelling and shrinkage (Zhang & Bourque, 2003). Since MNCs are osmoreceptors which rely on membrane tension to encode the ECF osmolality, the lack of volume compensatory processes are thought to allow SICs to transduce the changes in ECF osmolality to changes in electrical activity and thus hormone output (Zhang & Bourque, 2003).

1.2.1.1 Activation of PLC and changes in PIP₂ levels

The plasma membrane is made of many phospholipids, including phosphatidylinositol 4,5-bisphosphate (PIP₂), which is an important precursor to other second messenger molecules (Hokin, 1985; Raucher *et al.*, 2000; Boron & Boulpaep, 2008). Phospholipase C (PLC) is a membrane-associated protein that cleaves PIP₂ into inositol 1,4,5-triphosphate (IP₃) and diacylglycerol (DAG) (Wong *et al.*, 2005; Boron & Boulpaep, 2008). The DAG remains in the membrane and the IP₃ diffuses to the smooth endoplasmic reticulum (SER) to act on IP₃ receptors to release Ca²⁺ stores (Wong *et al.*, 2005; Boron & Boulpaep, 2008). This combination of a rise in [Ca²⁺]_i with DAG activates protein kinase C (PKC), which via phosphorylation causes modulation of many cellular events (Bell, 1986). Besides being a precursor molecule, PIP₂ is well known to anchor cytosolic proteins (McLaughlin *et al.*, 2002), regulate over 30 ion channels (Suh & Hille, 2008), and to have roles in cytoskeletal functions (Raucher *et al.*, 2000; Logan & Mandato, 2006) and membrane trafficking (Hilgemann *et al.*, 2001).

There are three groups of isoforms of PLC, PLC β , PLC γ and PLC δ , all of which have a range of requirements and mechanisms of activation that are specific to their respective activities (Rohacs *et al.*, 2008). The most evolutionarily conserved isoform group, PLC δ , is most poorly understood and not all animal groups have this specific isoform (Rebecchi & Pentyala, 2000; Irino *et al.*, 2004; Rohacs *et al.*, 2008). The regulation of the PLC δ isoforms is still under investigation and activation requires increased $[Ca^{2+}]_i$ (Allen *et al.*, 1997; Rohacs *et al.*, 2008). It is known that all PLC isoforms requires Ca^{2+} but the PLC δ s are particularly sensitive to small variations in physiological $[Ca^{2+}]_i$ (Allen *et al.*, 1997; Rohacs *et al.*, 2008). The second group of PLC isoforms include PLC β , which are activated by GPCRs via activation of G $\beta\gamma$ or Gq α subunits (Rebecchi & Pentyala, 2000; Rohacs *et al.*, 2008). Many hormones and bioactive molecules such as VP, bradykinin and acetylcholine act via this pathway to activate PLC β_{1-4} isoforms (Rebecchi & Pentyala, 2000; Rohacs *et al.*, 2008). Finally the PLC γ isoforms are activated by receptor tyrosine kinases, which are receptors for hormones such as insulin and nerve growth factor (Rebecchi & Pentyala, 2000; Rohacs *et al.*, 2008).

PIP₂ is particularly important in the regulation of voltage-gated K⁺ channels (Suh & Hille, 2002; Zhang *et al.*, 2003; Suh & Hille, 2005), VGCCs (Hille, 1994; Ikeda, 1996; Wu *et al.*, 2002; Suh & Hille, 2005) and TRP channels (Hilgemann *et al.*, 2001; Moran *et al.*, 2004; Brauchi *et al.*, 2007; Rohacs, 2007; Rohacs *et al.*, 2008). The original model of the effect of PIP₂ on TRPV1 postulated that under normal conditions, PIP₂ is present on the cell membrane with the TRPV1 channel and tonically suppresses its activity (Chuang *et al.*, 2001). During events that result in PLC activation, the PIP₂ degrades and lifts its tonic suppression of the TRPV1 channel resulting in increased TRPV1 activity (Chuang *et al.*, 2001). When PIP₂ was added or when antibodies against PIP₂ were used, the TRPV1 channels were suppressed and potentiated respectively (Chuang *et al.*, 2001).

Similar studies investigating the role of PIP₂ regulation of other TRPs, such as TRPM5 (Liu & Liman, 2003), TRPM7 (Runnels *et al.*, 2002), TRPM8 (Liu & Qin, 2005) and TRPA1 (Corey *et al.*, 2004; Hirono *et al.*, 2004), showed that PIP₂ has an opposite role, and results in activation of these channels (Rohacs *et al.*, 2008). However, other studies on TRPV1 channels showed that in excised membrane patches, PIP₂ actually potentiates TRPV1 (Stein *et al.*, 2006; Zhang & McNaughton, 2006; Lukacs *et al.*, 2007). This finding was later confirmed in the same excised patch preparation and showed that the inhibitory or activation response depends upon the concentration of TRPV1 agonists such as capsaicin (Lukacs *et al.*, 2007). Similar inconsistencies of other TRP channels have documented the dual modulatory role of PIP₂ on TRPA1 (Bautista *et al.*, 2006; Kwan *et al.*, 2006) and TRPC channels (Putney Jr, 2007).

Muscarinic GPCRs acting via PLC-mediated degradation of PIP₂ cause a decrease in the M-type K⁺ current (Suh & Hille, 2002; Zhang *et al.*, 2003; Ford *et al.*, 2004). This increases neuronal excitability by muscarinic activation of PLC, showing that PIP₂ has an activating role for those K⁺ channels (Zhang *et al.*, 2003; Ford *et al.*, 2004). The decrease in the M-current took about 10 seconds and recovered after 100-200 seconds, which is about the time it takes to degrade PIP₂ and resynthesize it, respectively (Suh *et al.*, 2004). The direct addition of PIP₂ was shown to activate these K⁺ channels (Zhang *et al.*, 2003; Ford *et al.*, 2004).

1.2.2 Long-term adaptations

Under chronic conditions of osmotic stress, the MNCs can undergo certain adaptations that allow them to sustain hormonal output (Hatton, 1997). The adaptations seen in MNCs fall under two broad categories, structural and functional (Hatton, 1997). These adaptive processes are recruited during the stress and are sustained for up to hours or days, but are reversed when the conditions

return to normal (Hatton, 1997). Current understandings and recent insights into this remarkable plasticity seen in MNCs will be further examined.

1.2.2.1 Structural adaptations

The MNCs and astrocytes of the SON undergo structural changes that may contribute to sustained hormonal output during chronic hypertonic conditions (Hatton, 1997). Normally, only one percent of the MNC membranes are in contact with other MNCs and the mean distance between them is about 10 nm (Tweedle & Hatton, 1977; Modney & Hatton, 1989; Marzban *et al.*, 1992). When the rat is dehydrated overnight, the MNC intercellular contact increases 10 times, which is reversible after rehydration (Tweedle & Hatton, 1976, 1977; Gregory *et al.*, 1980). In more extreme cases such as drinking hypertonic saline and dehydration lasting several days (7-10 days), results show that there exists a proportional relationship between amount of hyperosmotic stress and amount of glial withdrawal and soma hypertrophy (Modney & Hatton, 1989; Marzban *et al.*, 1992; Theodosis & Poulain, 1993). When hypertonic infusions are utilized, the glial retraction can be visualized after 5 hours (Beagley & Hatton, 1992; Hatton, 1997, 2002) and even after 30 minutes if infused transcardially (Tweedle *et al.*, 1993). Under hypotonic conditions, the amino acid taurine is released from the glia, which inhibits MNC firing and lowers VP output resulting in diuresis (Hussy *et al.*, 1997; Deleuze *et al.*, 1998; Deleuze *et al.*, 2005). The somatic area is shown to increase about 170% in electron micrographs of rat SONs subject to 10 day dehydration with salinized drinking water (Modney & Hatton, 1989). Capacitance measurements to measure changes in membrane surface area have confirmed a 33% increase in dehydrated rats with salinized drinking water (Tanaka *et al.*, 1999). In our lab, acutely isolated MNCs exposed to hypertonic solution for 90 minutes had a 7% increase in whole-cell capacitance (Shah *et al.*, 2014).

Due to the differences in hormonal output of the two types of MNCs, different stressors trigger different responses between both types (Bourque *et al.*, 1994; Bourque & Oliet, 1997; Bourque, 1999, 2008). During long term hypertonic stress, both VP and OT MNCs undergo the aforementioned changes, while pregnant rats undergoing lactation and parturition show the described structural changes only in OT MNCs (Hussy, 2002). A study documenting the effect of chronic hypotonicity on MNC structure has shown MNC shrinkage (Zhang *et al.*, 2001). The study investigated the cell and nuclear sizes of SON neurons and found that under chronic hypotonic conditions (about 223 mosmol/kg) for 7 days resulted in a 40% decrease in both cell soma and nuclear size (Zhang *et al.*, 2001). Thus the MNCs are structurally adaptable to both hypertonic and hypotonic osmotic stresses (Hatton, 1997).

1.2.2.2 Functional adaptations

Other changes in MNCs include the upregulation of specific ion channels and receptors on the cell membrane and also various genes to help MNCs to metabolically adapt in the long term (Glasgow *et al.*, 2000; Burbach *et al.*, 2001; Yue *et al.*, 2006). These functional changes, including changes in membrane and cytosolic proteins, are thought to lower electrical thresholds of the MNCs to facilitate phasic firing (Glasgow *et al.*, 2000; Burbach *et al.*, 2001; Yue *et al.*, 2006). These changes eventually result in changes in the cell activity and electrical firing patterns that help to sustain hormonal output (Hatton, 1997). The following sections will address some of the changes seen membrane and cytosolic proteins during chronic dehydration states.

1.2.2.3 Changes in cell surface levels of channels and receptors

During chronic conditions of hypertonicity, MNCs undergo a functional adaptation, including upregulation of many cell membrane channels and receptors (Shuster *et al.*, 1999; Tanaka *et al.*,

1999; Hurbin *et al.*, 2002; Tobin *et al.*, 2011). VP production and release is increased in response to chronic hypertonicity and this also causes autoreceptors in the SON to adjust to the increased VP output (Bourque, 2008). In a rat model, the mRNA of V_{1a} receptors decreased with increased water load and in opposition, during dehydration, the V_{1a} receptor levels were higher (Hurbin *et al.*, 2002). This co-expression of VP and its autoreceptor V_{1a} means that the nuclear pathways resulting in their synchronized regulation are similar or interconnected (Hurbin *et al.*, 2002). Given that antagonizing V_{1a} receptors leads to phasic firing, the increase in V_{1a} can be functionally relevant to modulate MNC electrical firing during high VP release (Hurbin *et al.*, 2002).

In another study, rats were salt-loaded and it was observed that the mRNA of α -II, Na6, β 1 and β 2 Na^+ subunits were increased (Tanaka *et al.*, 1999). In addition, immunofluorescence studies showed enhanced levels of Na^+ channels with electrophysiological confirmation of increased Na^+ transients and enhanced amplitude and density of persistent Na^+ currents (Tanaka *et al.*, 1999). Thus MNCs, in addition to increasing electrical activity through activation of Na^+ channels, have the adaptive ability to synthesize and translocate Na^+ channels to the plasma membrane and change its intrinsic electrical properties (Tanaka *et al.*, 1999).

Somatodendritic release in MNCs is a very dynamic process and involves other peptides co-released with VP to help ensure regulation of hormone output (Shuster *et al.*, 1999; Brown & Bourque, 2004, 2006). It is shown that salt-loading causes an increase in VP release that was concomitant with a rise in κ -opioid receptors at the plasma membrane (Shuster *et al.*, 1999). More specifically, immunofluorescence studies showed that the ratio between κ -opioid receptor 1 (κ OR1) in the plasma membrane and in vesicles containing neuropeptides significantly increased with hypertonic stimulus (Shuster *et al.*, 1999). Dynorphin is released with VP and decreases Ca^{2+} influx and neuropeptide release (Brown & Bourque, 2004, 2006). Thus the co-release of dynorphin

along with an increase in κ OR1 on the plasma membrane results in an increase in autocrine-like regulation and decrease in VP and OT release (Shuster *et al.*, 1999; Brown & Bourque, 2004, 2006).

1.2.2.4 Changes in cellular protein expression

During conditions of chronic osmotic stress, the MNCs undergo specific proteomic changes (Glasgow *et al.*, 2000; Burbach *et al.*, 2001; Yue *et al.*, 2006). The studies documenting the effects of gene upregulation in hypertonic treated rats show that 37 genes are upregulated from hypertonic treated rats compared to hypotonic treated rats (Glasgow *et al.*, 2000). Some of the over-expressed genes include so-called “housekeeping” genes, which are involved in various cellular processes such as energy metabolism and cell homeostasis (Glasgow *et al.*, 2000). The Na^+/K^+ -ATPase is one such protein that is upregulated and is involved in three main processes, maintenance of resting membrane potential, driver of secondary active transport and maintenance of cell volume (Geering, 1997). Another common protein found in the mitochondria, the cytochrome-*c* oxidase is also upregulated, which suggests that the cell is preparing for increased protein expression by increasing the mitochondrial energy production capacity (Krukoff *et al.*, 1983; Wong-Riley, 1989; Glasgow *et al.*, 2000). This study also documented the increase in expression of PEP-19, which is a calmodulin-binding protein (Glasgow *et al.*, 2000). Increased expression of PEP-19 is thought to decrease the amount of Ca^{2+} -mediated and calmodulin associated cellular pathways (Gainer & Wray, 1994; Glasgow *et al.*, 2000). Thus in chronic osmotic stress events the increase in $[\text{Ca}^{2+}]_i$ could be dangerous to MNCs and the increase in PEP-19 could act as a safeguard (Gainer & Wray, 1994).

1.2.2.5 Translocation of channels and receptors in other neurons

Exocytosis involves fusion of smaller vesicles within the cell to the plasma membrane to release its inner contents, which may include hormones, neurotransmitters or small bioactive molecules (Morgan, 1995). During release of the hormones or neurotransmitters in neurons, the action potentials generated cause activation of VGCCs which allows Ca^{2+} influx, exocytotic fusion of the neuropeptide vesicles and release into the extracellular space (Hatton, 1990; Armstrong, 1995). There also exists a fusion machinery that allows interaction, docking and fusion of the synaptic vesicles with the plasma membrane (Pryer *et al.*, 1992; Rothman, 1994; Sudhof, 1995).

Constitutive exocytosis is an unregulated pathway that cells use for delivery to the cell membrane for constant extracellular release (Morgan, 1995; Gerber & Südhof, 2002), while regulated exocytosis involves intracellular control of the plasma membrane fusion events, mainly executed by secretory cells and neurons (Burgoyne & Morgan, 1993). Regulated exocytosis can involve translocation, a transfer of receptors and channels to the cell surface that can change the cellular activity (Chieriegatti & Meldolesi, 2005). In kidneys, AQP2 is translocated to the plasma membrane through VP-mediated action on the V_2 receptor (Agre, 2006; Petersen, 2006; Ball, 2007). A classical neuronal example occurs during learning and memory with the receptors involved in the synaptic plasticity of hippocampal neurons (Malinow & Malenka, 2002). Long term potentiation (LTP) in these cells involves insertion of GluR1, the subunit of the α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor (AMPA) (Malinow & Malenka, 2002). This translocation of the GluR1 subunit involves increased $[\text{Ca}^{2+}]_i$, calmodulin and vesicle-associated membrane protein-2 (VAMP2), a controller of regulated exocytosis (Hayashi *et al.*, 2000; Lu *et al.*, 2001). In OT MNCs, using thapsigargin to increase $[\text{Ca}^{2+}]_i$, resulted in an increase in N-type Ca^{2+} current which is thought to be due to translocation of the N-type Ca^{2+} channels to

the plasma membrane (Tobin *et al.*, 2011). Other receptors that are translocated upon exogenous stimulation include γ -aminobutyric acid-A (GABAA) (Wan *et al.*, 1997) and NMDARs (Lan *et al.*, 2001). Stimulation with growth factors can cause rapid translocation of TRPC5 and TRPC3 channels in neurons (Clapham, 2003; Bezzerides *et al.*, 2004; Singh *et al.*, 2004). Thus the phenomenon of translocation in neurons has an effect to aid functional adaptations to facilitate events such as synaptic plasticity, electrical excitability, sensitivity to exogenous mediators and axonal growth (Burgoyne & Morgan, 1993; Chieriegatti & Meldolesi, 2005; Cocucci *et al.*, 2006).

The translocation of channels and receptors is a dynamic process and can involve reuptake during conditions when those receptors and channels aren't required (Vieira *et al.*, 1996; Carroll *et al.*, 2001). This is referred to endocytosis, which requires a separate set of molecular machinery (Goldstein *et al.*, 1979; Pastan & Willingham, 1985). Vesicles are formed by a class of coat proteins called clathrin that forms invaginations at the plasma membrane with the assistance of several proteins involved in the endocytosis process (Le Roy & Wrana, 2005; McMahon & Boucrot, 2011). A final protein known as dynamin, via hydrolysis of guanosine-5'-triphosphate (GTP), is required for the final pinching off of the invaginations and formation of the vesicle (Boron & Boulpaep, 2008; Morlot & Roux, 2013).

1.2.2.6 PKC involvement in translocation

Regulated exocytosis involves the same basic mechanisms for vesicular fusion as constitutive exocytosis, but the process also includes other proteins involved in fine-tuning the temporal and spatial control of these events (Morgan *et al.*, 2005). Many studies have documented protein kinase A and PKC in the regulated exocytosis of secretory and neuronal cells (Lindau & Gomperts, 1991; Hille *et al.*, 1999; Turner *et al.*, 1999; Leenders & Sheng, 2005). PKC requires the combination of

phosphatidylserine, DAG and Ca^{2+} for maximal activity, and is differentiated into isoenzyme families based on their specific requirements for activity (Newton, 2001). The role of PKC in exocytosis was discovered by observations that phorbol esters (analogues of DAG) stimulated secretion from cells (Terbush & Holz, 1990). The DAG was confirmed to act via PKC, as co-administering PKC inhibitors with phorbol esters negated the enhanced secretory response (Terbush & Holz, 1990). A study that added purified PKC enzyme in permeabilized chromaffin cells saw increased exocytosis, showing PKC is regulating the exocytotic machinery with Ca^{2+} as a cofactor (Naor *et al.*, 1989; Morgan & Burgoyne, 1992).

The role of PKC in the functional adaptation of cells has been well documented and strengthens its role as a mediator of non-secretory exocytosis. It has been shown that stimulation of PKC in *Aplysia* bag cell neurons causes an increase in Ca^{2+} currents and that the mechanism includes translocation of specific Ca^{2+} channels (Strong *et al.*, 1987). In an oocyte expression system, exogenous activation of PKC resulted in a fast translocation of TRPV1 channels to the plasma membrane (Morenilla-Palao *et al.*, 2004). This rapid increase of TRPV1 on the plasma membrane was blocked with botulinum neurotoxin A showing that the phenomenon is indeed a translocation event occurring through soluble *N*-ethylmaleimide sensitive fusion (NSF) attachment protein receptor (SNARE)-mediated exocytosis (Morenilla-Palao *et al.*, 2004). Taken together, these data show that PKC can cause translocation of ion channels and receptors, which ultimately leads to changes in cellular phenotype (Strong *et al.*, 1987; Naor *et al.*, 1989; Terbush & Holz, 1990; Morgan & Burgoyne, 1992; Newton, 2001; Morenilla-Palao *et al.*, 2004; Morgan *et al.*, 2005).

1.3 Recent findings from the lab

Our lab focuses on the structural and electrophysiological adaptations of MNCs under chronic hypertonic stress. Using biochemical and electrophysiological assays we are attempting to determine the mechanism to understand how the MNCs adapt. This section will review some of our most recent observations and our current and future directions.

The cellular events underlying the MNC hypertrophy are difficult to elucidate in the above mentioned *in vivo* models. Our lab has recently developed an *in vitro* model to use various pharmacological and electrophysiological tools to assess and better understand the hypertrophy (Shah *et al.*, 2014). I will now briefly mention some of the findings from previous members of the lab.

When acutely isolated MNCs are subject to a 30 mosmol/kg increase in hypertonicity, they undergo shrinkage (as most cell types do) but over the course of 150 minutes undergo hypertrophy to about 105% of their control size (Shah *et al.*, 2014). This hypertrophy effect is reversible and does not occur in hippocampal neurons (Shah *et al.*, 2014). To investigate cellular activities involved in hypertrophy various pharmacological tools were utilized. Hypertrophy can be blocked by pre-treatment with tetrodotoxin (TTX), SIC channel blocker (SB366791), nifedipine and BAPTA-AM (Shah *et al.*, 2014). These results suggest that hypertrophy is dependent upon action potentials, SIC activity, increased $[Ca^{2+}]_i$, and Ca^{2+} influx through L-type Ca^{2+} channels (Shah *et al.*, 2014). The hypertrophy effect can also be reversed by addition of TTX, SB366791 and nifedipine suggesting its maintenance depends on continued electrical activity and sustained Ca^{2+} entry (Shah *et al.*, 2014). The mechanism of cell enlargement is thought to be through insertion of

new plasma membrane by exocytotic fusion because a blocker of SNARE-dependent exocytotic fusion prevents the hypertrophy (Shah *et al.*, 2014).

It was shown in our lab recently that osmotic stimuli (increases in external osmolality) leads to an activation of PLC and subsequent decreases in PIP₂ (Shah *et al.*, 2014). Under control conditions, it was seen that acutely isolated MNCs have a strong and membrane-selective immunoreactivity (Shah *et al.*, 2014). A five minute exposure to 325 mosmol/kg saline resulted in an approximately 30% decrease in membrane PIP₂ immunoreactivity (Shah *et al.*, 2014). Further, preincubation of the cells with a PLC inhibitor prevented osmotically-induced changes to PIP₂ immunoreactivity. Adding a muscarinic receptor agonist (resulting in PLC activation) caused a similar decrease in PIP₂ immunoreactivity that could be blocked by the PLC inhibitor U73122 (Shah *et al.*, 2014). This finding shows that short term exposure of MNCs to high osmolality causes osmotic activation of PLC, which then reduces levels of PIP₂ (Hilgemann *et al.*, 2001; Shah *et al.*, 2014). A very similar finding has been seen in green algae, which shows that hyperosmotic stress causes an increase in phospholipase D activity within minutes of hypertonic exposure and also depends on the size of the increase in osmolality (Munnik *et al.*, 2000a). Both of these findings are seen in our *in vitro* rat MNC model documenting changes in PLC activity (Shah *et al.*, 2014).

A rise in [Ca²⁺]_i is the main cue for hormonal and neurotransmitter output from many secretory cells such as endocrine cells and neurons respectively. Recent findings from our lab show that specifically, L-type Ca²⁺ currents increase (77%) in the SON of rats that have been deprived of water (Zhang *et al.*, 2007a). Radioligand binding assays suggest that rather than a modulation of channel function, there is an increase in the abundance of channels on the membrane (32%) through translocation of channels from reserves or via increase in protein translation of existing α_1

subunits (Zhang *et al.*, 2007a). The latter explanation of increased protein translation seems plausible as there is no increase in mRNA of L-type Ca^{2+} subunits (Zhang *et al.*, 2007a). The increase in L-type Ca^{2+} currents may be important in modulating electrical activity through DAP and AHP currents (Andrew & Dudek, 1983; Armstrong *et al.*, 1994; Li *et al.*, 1995). Since it is known that L-type Ca^{2+} currents are involved in activating CREB, they may be of importance in increasing expression of genes (Burbach *et al.*, 2001).

CHAPTER 2: HYPOTHESIS AND OBJECTIVES

It is difficult to elucidate the mechanism of dehydration-induced MNC hypertrophy using *in vivo* models because of the difficulty in selectively targeting MNCs pharmacologically. Previous findings using an *in vitro* model of acutely isolated MNCs led to the conclusion that osmotically-evoked hypertrophy to 105% of control size occurs *in vitro* in response to 325 mosmol kg⁻¹ (Shah *et al.*, 2014). The osmotically-evoked hypertrophy involves insertion of new membrane via SNARE-mediated exocytotic fusion as evident by a 7% increase in whole-cell capacitance (Shah *et al.*, 2014). The initiation and maintenance of hypertrophy depends upon action potentials, SIC activation, increased [Ca²⁺]_i and Ca²⁺ influx through L-type Ca²⁺ channels (Shah *et al.*, 2014). Finally, MNCs from rats that have been deprived of water show an 80% increase in L-type Ca²⁺ current density and radioligand binding assays show a 32% increase in L-type Ca²⁺ channel density (Zhang *et al.*, 2007a).

Based on these conclusions we designed the following hypotheses using our *in vitro* model to further study osmotically-induced hypertrophy in acutely isolated MNCs.

- 1.) Osmotically-evoked hypertrophy occurs physiologically in a dose-dependent manner, such that smaller increases in osmolality result in proportionally smaller amounts of hypertrophy.
- 2.) Osmotically-evoked hypertrophy occurs independently of cell volume regulatory processes and does not depend on activation of NKCC1.
- 3.) Reversal of hypertrophy depends upon dynamin-mediated endocytosis.
- 4.) Osmotically-evoked hypertrophy depends upon activation of PLC and PKC.
- 5.) Hypertrophy can be induced by neuronal depolarization, Ca²⁺ entry and activation of PKC.

- 6.) Sustained exposure to hypo-osmolar solutions results in a reversible and sustained decrease in MNC soma size.
- 7.) Sustained exposure to hypertonic solutions will increase immunolabeling of the L-type Ca^{2+} channel $\text{Cav}1.2$ subunit.

The neurohormonal mechanisms underlying the regulation of extracellular osmolality are of critical physiological importance (Bourque, 2008). These mechanisms act to maintain the osmolality of human plasma close to a “set-point” of 290 milliosmoles per kilogram (Bourque, 2008). The primary hormonal regulator of osmolality is vasopressin (VP), which is released by the magnocellular neurosecretory cells (MNCs) of the SON in the hypothalamus as a function of plasma osmolality (Bourque *et al.*, 1994; Bourque & Olier, 1997; Bourque, 2008). The objectives of this thesis are to understand the mechanisms involved in osmotically-induced hypertrophy and increased expression of L-type Ca^{2+} channels in MNCs exposed to sustained increases in osmolality as seen in the elderly and chronically ill (O'Neill *et al.*, 1990). The proposed project will help to elucidate the mechanisms underlying this important example of neuroendocrine adaptation and help to understand the regulation of body fluid balance during chronic challenges.

CHAPTER 3: MATERIALS AND METHODS

3.1 Chemicals

All chemicals, unless stated otherwise, were from Sigma-Aldrich Corporation (St. Louis, MO, U.S.A.). The membrane dye CellMask™ Orange was purchased from Invitrogen (Carlsbad, CA). The primary antibody, Cav1.2 was from Alomone Labs (Jerusalem, Israel). The secondary antibodies were Alexa fluor 488 anti-rabbit from Invitrogen (Carlsbad, CA).

3.2 Animals and cell preparation

The handling of the animals and the experiments were approved by the University of Saskatchewan's Animal Research Ethics Board and adhered to the Canadian Council on Animal Care guidelines for humane animal use. Male Long Evans rats weighing 250-300 grams were given *ad libitum* food and water and were used in all of the described experiments. The previously established MNC isolation protocol (Liu *et al.*, 2005) and the criteria for MNC identification were followed (Oliet & Bourque, 1992). Briefly, the rats were placed in a chamber containing halothane for anaesthetization and a small animal guillotine was used for decapitation. The skull was opened and the brain was hydrated with a PIPES (piperazine-N,N'-bis(2-ethanesulfonic acid)) buffer solution composed of (in mM, pH 7.10): NaCl, 110; KCl, 5; MgCl₂, 1; CaCl₂, 1; PIPES, 20 and glucose, 25 (abbreviated as PGC). The osmolality of the isotonic PGC was maintained at 295 ± 3 mosmol kg⁻¹ with a VAPRO vapor pressure osmometer (Wescor, Logan, UT, U.S.A.). The hypertonic osmolalities were obtained by adding mannitol to isotonic PGC. Hypotonic PGC (280 mosmol kg⁻¹) was made by adding 10 mM glucose (instead of 25 mM) to PIPES. Upon hydration, an approximately 1.0 mm thick coronal slice of the brain was removed that contained most of the

SON and pinned to a petri dish submerged in isotonic PGC. Tissue blocks containing SON were excised and placed in isotonic PGC containing trypsin from bovine pancreas type XI, 0.6 mg/mL), bubbled with 100% O₂ in a water bath maintained at 34°C for 90 minutes. The tissue blocks were then transferred to non-trypsinized PGC, and were bubbled with 100% O₂ at room temperature for 30 minutes. The blocks were then mechanically dispersed by trituration using fire-polished Pasteur glass pipettes with graded decreases in opening diameter. The triturated MNCs were plated onto glass bottom culture dishes, covered to avoid evaporation, and allowed to adhere to the glass for 30 minutes at room temperature before initiating experiments.

3.3 Morphometry experiments

Acutely isolated MNCs were mounted onto the microscope stage and oxygenated PIPES solutions of various osmolalities were perfused into the plate using a gravity perfusion system. In some experiments, MNCs were exposed to stationary baths (no perfusion) of specific osmolalities, with or without chemicals, as indicated in the text. On average, there were 2-3 MNCs per frame of view in each experiment. The MNCs were photographed with a cooled charge-coupled device (CCD) camera attached to a Zeiss Axiovert 200 microscope with a 40x objective at various indicated times throughout the experiments. The maximal circumference of the cell soma was traced and the cross-sectional area (CSA) was determined using *ImageJ* (National Institutes of Health). The data was normalized by dividing the CSA of each image with the average CSA of the same cell under control conditions while in isotonic PGC and are represented as mean \pm standard error of mean (SEM). Only the cells that showed a hypertrophy response (shrinkage, enlargement and recovery of cell soma) were used in forming the graphs. Cells which did not recover back to their control CSA were deemed unhealthy and so were excluded.

3.4 Fluorescence images of hypertrophy

Acutely isolated MNCs were stained with the plasma-membrane specific stain, CellMask™ Orange (Invitrogen). Briefly, after plating the MNCs and allowing them to adhere, the cells were incubated with 5 µg/mL CellMask™ Orange for 5 minutes and subsequently rinsed with isotonic saline three times. The cells were imaged with appropriate filter sets first in isotonic saline for control images and then treated with hypertonic saline for subsequent images at 5 and 80 minutes.

3.5 Immunocytochemistry of Cav1.2 subunit

Acutely isolated MNCs were exposed to isotonic PGC, hypertonic PGC or hypertonic PGC + TTX (1 µM) for 120 minutes after which they were washed three times with phosphate buffered saline (PBS). The MNCs were then washed three times with 10% formalin solution for 20 minutes at room temperature. Subsequently the cells were washed three times with PBS. The MNCs were then treated with blocking solution (10% donkey serum with Triton-X in PBS) for one hour at room temperature. The MNCs were then incubated with primary antibodies from Alomone Labs against the L-type Ca^{2+} subunit Cav1.2 (1:500) at 4°C overnight. The negative controls used in the experiments were not incubated with primary antibody. The following morning, the MNCs were washed three times with PBS and incubated in the secondary antibody, Alexa fluor 488 donkey anti-rabbit (1:2000) from Invitrogen. The MNCs were given a final three washes. The mounting solution, Citifluor, was added to the wells of each plate and the cells were viewed with a Zeiss Axiovert 200 inverted microscope with a 40x objective with differential image contrast (DIC) and fluorescence image filter sets. The images were captured using a CCD camera and *ImageJ* software was used to measure the mean fluorescence intensity by drawing a perimeter around the cell soma.

The data was normalized to the mean fluorescent intensity of all cells treated with isotonic PGC on the same day and represented as mean fluorescence \pm SEM.

3.6 Data analysis

The data was entered into GraphPad Prism® and various statistical tests were used, as indicated, to determine statistical significance. All results are shown as mean \pm SEM. A statistical test showing with a confidence level of $P < 0.05$ was deemed statistically significant.

CHAPTER 4: RESULTS

4.1 Osmotically-evoked hypertrophy occurs at physiological osmolalities in a dose-dependent manner

Previous members of the lab have shown that MNCs exposed to hypertonic solution display an immediate shrinkage but undergo reversible hypertrophy over the course of 150 minutes (Shah *et al.*, 2014). We wanted to see if hypertrophy can occur at more physiological increases of hypertonicity and thus see if the effect size is osmolality-dependent. Acutely isolated MNCs were perfused with 100% oxygenated isotonic PGC (295 mosmol kg⁻¹) for 10 minutes to record the basal control CSA. The MNCs (n=12; 4 experiments) were then subsequently perfused with hypertonic saline (325 mosmol kg⁻¹) and the CSA was monitored at various time points. Figure 4.1 represents an MNC fluorescently labeled with a membrane-selective dye (CellMask) undergoing both shrinkage (at 5 minutes) and hypertrophy (80 minutes). The red line is the control CSA and represents shrinkage (red line encircling the cell perimeter) and hypertrophy (red line smaller than cell perimeter). As shown in Figure 4.2, the MNCs shrunk to a CSA of $94.5 \pm 0.8\%$ of control (a mean CSA reduction from $363.4 \pm 36.3 \mu\text{m}^2$ to $343.4 \pm 35.4 \mu\text{m}^2$) after 5 minutes and after about 20 minutes started to hypertrophy and peaked in size at $105.2 \pm 0.7\%$ ($381.7 \pm 38.2 \mu\text{m}^2$) after about 60 minutes. The mean shrunken and enlarged CSA (measured at 5 and 60 minutes, respectively), were significantly different than the mean CSA of the control (using a one-way repeated measures analysis of variance test; $P < 0.01$ in both cases). The amount of hypertonicity was reduced to see if osmotically-evoked hypertrophy occurs at more physiological hypertonic osmolalities. When the MNCs were exposed to an osmolality of 305 mosmol kg⁻¹ (n=10; 2 experiments) the cells (from a baseline mean CSA of $357.2 \pm 30.1 \mu\text{m}^2$) had reduced levels of

shrinkage ($348.7 \pm 28.9 \mu\text{m}^2$; $97.6 \pm 0.4\%$) and hypertrophy ($368.1 \pm 29.9 \mu\text{m}^2$; $103.1 \pm 0.4\%$). When the MNCs were exposed to an osmolality of 300 mosmol kg^{-1} (n=10; 2 experiments) the cells has a baseline mean CSA of $324.9 \pm 25 \mu\text{m}^2$ and had even lesser levels of shrinkage ($319.5 \pm 25 \mu\text{m}^2$; $98.2 \pm 0.4\%$) and hypertrophy ($328.4 \pm 25.2 \mu\text{m}^2$; $101.1 \pm 0.2\%$). Each of the shrunken and enlarged CSAs of the MNCs from both lower hypertonic treatments (300 and 305 mosmol kg^{-1}) were significantly different than their control CSA using a one-way analysis of variance test ($P < 0.01$). In all hypertonic treatments, when the MNCs were returned to the control isotonic PGC, they rapidly returned to their control CSA after about 5-10 minutes. A control experiment involving MNCs (n=5; 1 experiment) perfused with isotonic PGC (295 mosmol kg^{-1}) throughout the experiment showed that there is no change in MNC soma size. The mean CSA of the shrunken and hypertrophied states of all three hypertonic treatments was shown to be statistically different than the mean CSA of MNCs treated with isotonic PGC at the same time periods (using a two-way analysis of variance; $P < 0.01$ in all cases). The amount of shrinkage seen in the hypertonic treated MNCs is comparable to the predicated amount of shrinkage in response to a short-term hypertonic shock (Zhang & Bourque, 2003). The results suggest that MNC hypertrophy is osmotically-evoked and dependent upon the degree of osmolality change with the ability to reverse its response as seen *in vivo* (Hatton, 1997; Tanaka *et al.*, 1999; Di & Tasker, 2004).

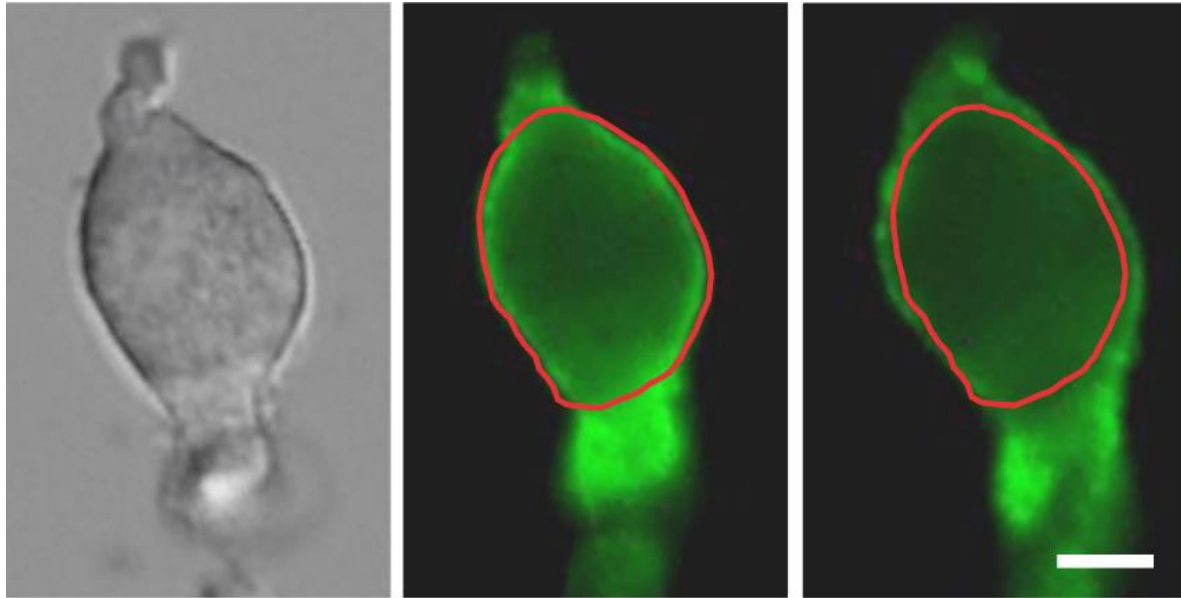


Figure 4.1: Fluorescence images of a MNC undergoing shrinkage and hypertrophy. The image on the left shows a DIC image of an isolated MNC in isotonic PGC. The other images shows the same MNC stained with a membrane dye (CellMask) at 5 minutes (middle) and 80 minutes (right) after exposure to hypertonic PGC. The red line shows the cellular perimeter under isotonic conditions for comparison. The perimeter of the cell in the middle is smaller than the control red line, while the perimeter of the cell on the right is larger than the control cell line.

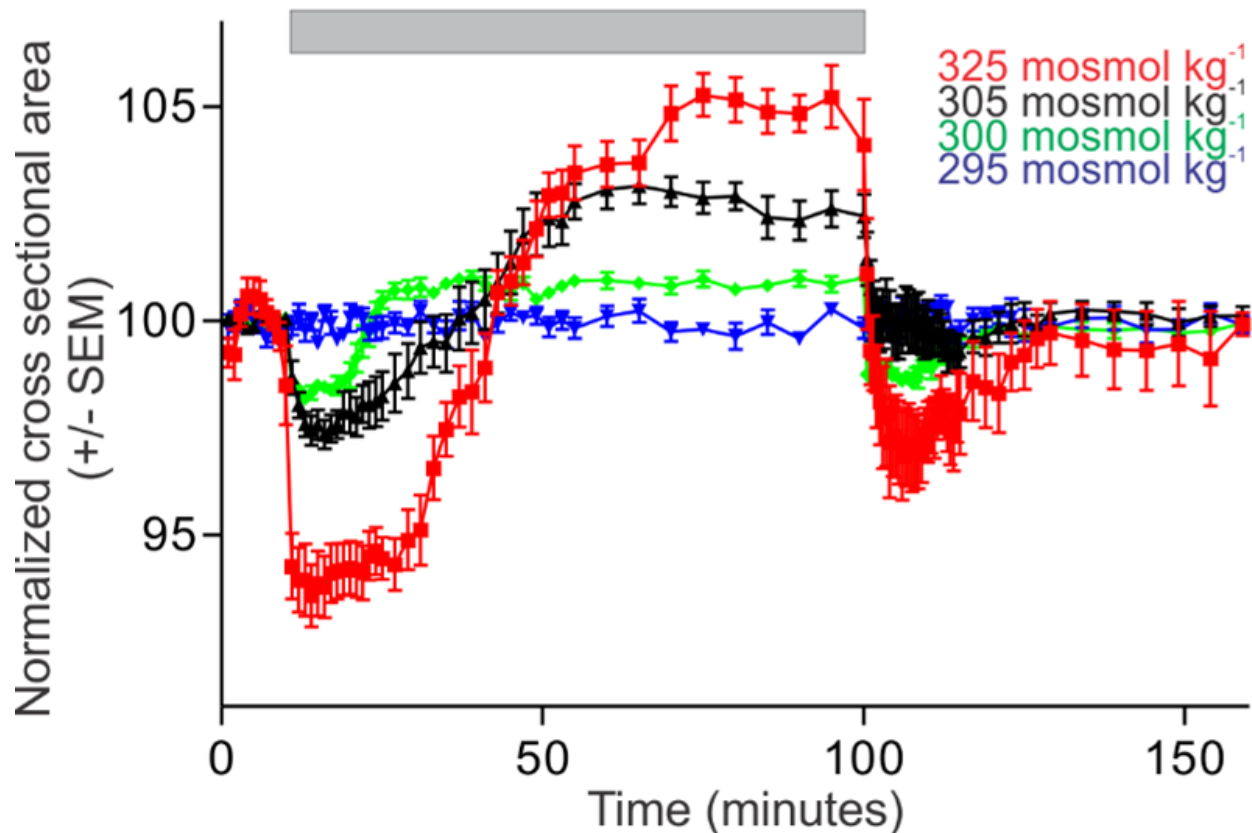


Figure 4.2: Increases in osmolality evoke reversible hypertrophy in osmosensitive MNCs of the SON in a dose-dependent manner. Perfusion of oxygenated hypertonic PGC (325, 305 and 300 mosmol kg⁻¹) causes acutely isolated MNCs to shrink and hypertrophy over 90 minutes (n=12, 10 and 10, respectively), while perfusion of isotonic PGC (295 mosmol kg⁻¹) results in no change in cell size. Returning the MNCs to isotonic PGC results in a rapid reversal of hypertrophy to the original control CSA. The period of hypertonic PGC perfusion is indicated by the grey bar at the top of the plot.

4.2 Osmotically-evoked hypertrophy occurs independently of NKCC1-mediated cell volume regulatory processes

We wanted to test whether hypertrophy requires activation of the $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ co-transporter NKCC1, which is commonly involved in cell volume regulatory processes (O'Neill & Klein, 1992; Nardou *et al.*, 2009). Acutely isolated MNCs were treated with hypertonic PGC in a stationary bath with the addition of bumetanide (10 μM), an antagonist of the NKCC1. Bumetanide at 10 μM is known to block NKCC1 effectively without affecting other membrane ionic transporters such as $\text{Cl}^-/\text{HCO}_3^-$ and Na^+/H^+ exchangers or the K-Cl cotransporter KCC2 (O'Grady *et al.*, 1987; Gillen *et al.*, 1996; Tyzio *et al.*, 2006). The cells were first incubated in isotonic PGC for 10 minutes followed by switching the stationary bath to 325 mosmol kg^{-1} hypertonic PGC with bumetanide. The MNCs CSA was monitored at various time points. As shown in Figure 4.3, the MNCs treated with bumetanide ($n=10$; 3 experiments) shrunk to a CSA of $94.4 \pm 0.7\%$ of control after 5 minutes and hypertrophied to a maximum CSA of $105.2 \pm 0.7\%$ after 75 minutes. The mean shrunk and enlarged CSA of bumetanide-treated cells (measured at 5 and 75 minutes, respectively), were significantly different than the mean CSA of the control (using a one-way repeated measures analysis of variance test; $P < 0.01$ in both cases). The mean CSA of the shrunk and hypertrophied states of the bumetanide-treated MNCs in hypertonic PGC were not statistically different than the mean CSA of MNCs treated with hypertonic PGC without bumetanide at the same time periods (using a two-way analysis of variance; $P > 0.05$ in both cases). When the MNCs were returned to the control isotonic PGC following hypertonic treatment with or without bumetanide, they rapidly returned to their control CSA after about 5-10 minutes. The hypertrophic response did not appear to be affected by the addition of bumetanide, suggesting NKCC1-mediated cell volume regulation seems to not be involved in osmotically-evoked hypertrophy.

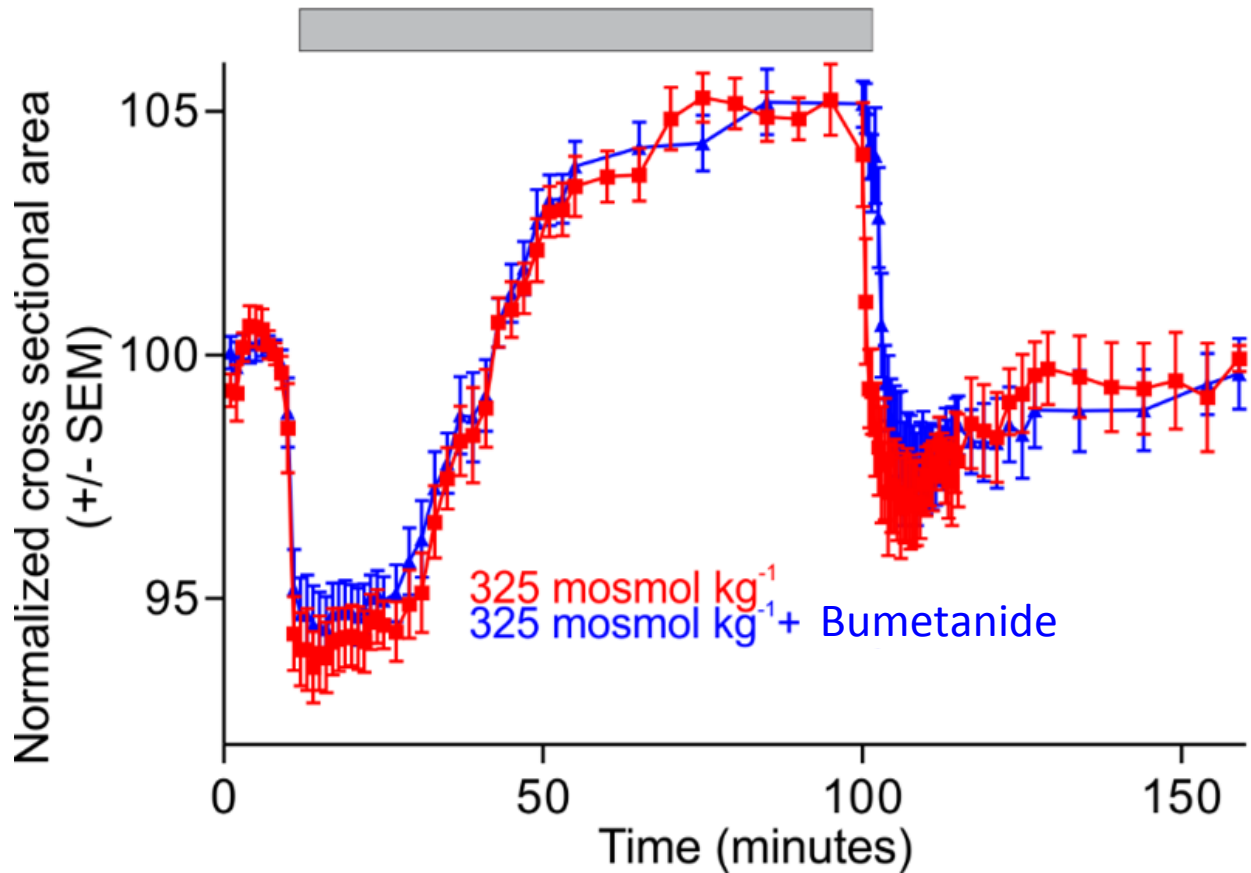


Figure 4.3: Osmotically-evoked hypertrophy does not require activation of NKCC1. The shrinkage and hypertrophy response of MNCs (n=10) to hypertonic saline (325 mosmol kg⁻¹) was not affected by the presence of bumetanide (10 μ M), which is an inhibitor of the Na⁺-K⁺-Cl⁻ co-transporter NKCC1. The response of the MNCs (n=12) to perfusion of hypertonic saline (325 mosmol kg⁻¹) without bumetanide is shown for comparison. Returning the MNCs to isotonic PGC results in a rapid reversal of hypertrophy to the original control CSA in both groups. The period of hypertonic PGC exposure is indicated by the grey bar on the top of the plot.

4.3 Reversal of hypertrophy depends upon dynamin-mediated endocytosis

We wanted to examine the role of dynamin-dependent endocytosis in the reversal of MNC hypertrophy. Acutely isolated MNCs were perfused with hypertonic PGC and they were incubated with dynasore (80 μ M), an inhibitor of dynamin-dependent endocytosis (Macia *et al.*, 2006; Kirchhausen *et al.*, 2008) prior to recovering the cells with isotonic PGC. The cells were first incubated in isotonic PGC for 10 minutes followed by perfusion with 325 mosmol kg^{-1} hypertonic PGC. The MNCs CSA was monitored at various time points. As shown in Figure 4.4, the MNCs treated with dynasore (n=10; 3 experiments) shrunk to a CSA of $94.7 \pm 0.5\%$ of control after 5 minutes and hypertrophied to a maximum CSA of $104.9 \pm 1\%$ after 85 minutes. The mean shrunken and enlarged CSA of dynasore-treated cells (measured at 5 and 85 minutes, respectively), were significantly different than the mean CSA of the control (using a one-way repeated measures analysis of variance test; $P < 0.01$ in both cases). However, the mean CSA of the MNCs after adding isotonic PGC for recovery was statistically different than the mean CSA of the control (using a one-way repeated measures analysis of variance test; $P < 0.01$). In addition, the mean CSA of the MNCs after adding isotonic PGC for recovery was not statistically different than the mean CSA of the maximal hypertrophied state (using a one-way analysis of variance test; $P > 0.05$). The results suggest that the hypertrophic response was unaffected by the addition of dynasore. However the MNCs were not able to recover following addition of isotonic PGC. The recovery of MNCs from osmotically-evoked hypertrophy therefore seems to involve dynamin-dependent endocytosis.

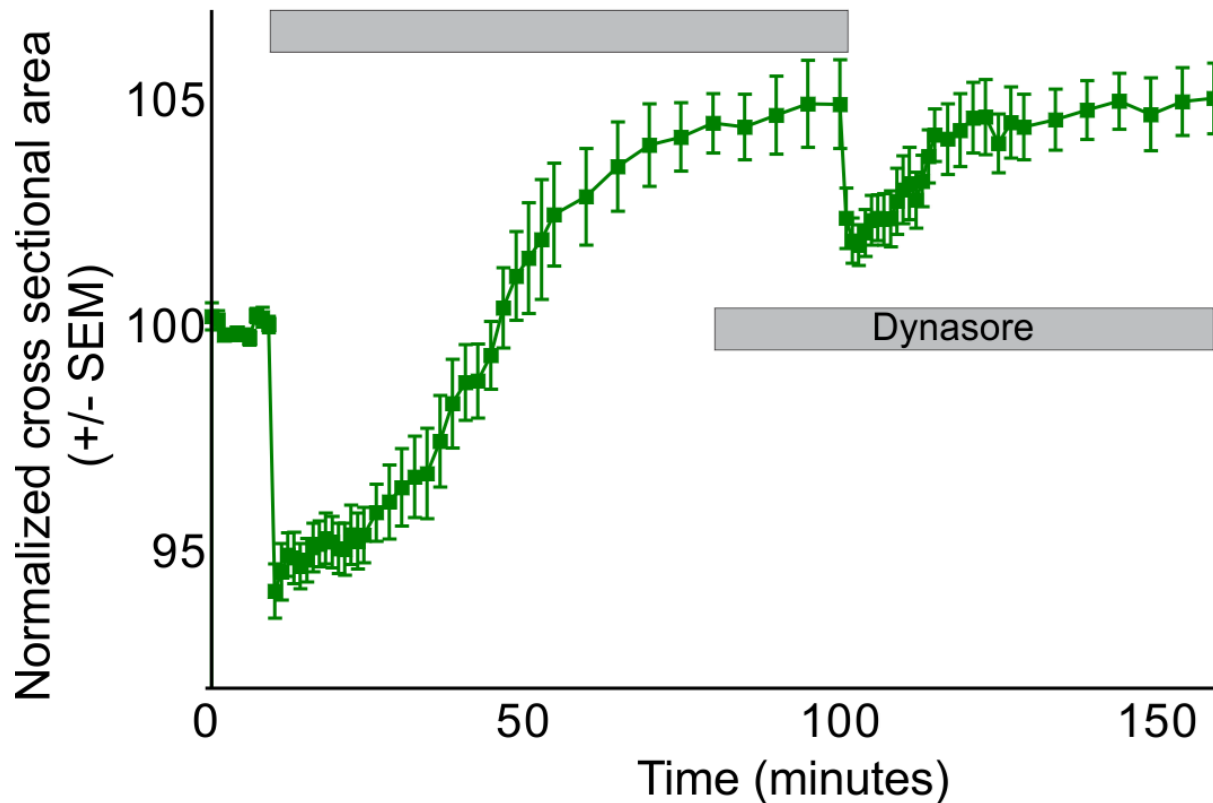


Figure 4.4: Reversal of osmotically-evoked hypertrophy depends on dynamin-dependent endocytosis. The shrinkage and hypertrophy response of MNCs (n=10) to hypertonic saline (325 mosmol kg⁻¹) was not affected by the presence of dynasore (80 μM), which is an inhibitor of dynamin-dependent endocytosis. The mean CSA of MNCs after perfusion with isotonic PGC was statistically different than the control CSA, but not statistically different than the maximal hypertrophied state. The period of hypertonic PGC exposure is indicated by the grey bar on the top of the plot and the exposure of the MNCs to dynasore is indicated by the lower grey bar.

4.4 The activation of PLC and PKC are requirements for osmotically-evoked hypertrophy

The role of PLC and PKC in translocation of channel and receptors have been studied in both neuronal and non-neuronal cells (Strong *et al.*, 1987; Van Epps-Fung *et al.*, 1997; Lin *et al.*, 2002; Morenilla-Palao *et al.*, 2004). We wanted to see whether the activation of PLC or PKC is required to undergo hypertrophy in MNCs. Acutely isolated MNCs were exposed to hypertonic PGC in the presence of an inhibitor of PLC (U73122; 1 μ M) or PKC (bisindolylmaleimide I; 1 μ M) to understand whether activation of either one or both enzymes is required for osmotically-evoked hypertrophy. The cells were first incubated in isotonic PGC for 5 minutes followed by addition of 325 mosmol kg⁻¹ hypertonic PGC with either enzyme inhibitor. The MNCs CSA was monitored at various time points. As shown in Figure 4.5, the MNCs treated with the PLC inhibitor (n=12; 4 experiments) shrunk to a CSA of $94.1 \pm 0.4\%$ of control and remained in the shrunken state, which was statistically different than the mean CSA of the control (using a one-way analysis of variance test; $P < 0.01$). The MNCs treated with the PKC inhibitor (n=12; 5 experiments) shrunk to a CSA of $95.1 \pm 0.3\%$ of control and remained in the shrunken state, which was statistically different than the mean CSA of the control (using a one-way analysis of variance test; $P < 0.01$). When both treatment groups were re-exposed to isotonic PGC, the MNCs rapidly recovered to their respective control size and their CSAs were not statistically different than the control CSAs (using a one-way analysis of variance test; $P > 0.05$ for both treatments). The results show the hypertrophic response was prevented by inhibitors of both PLC and PKC suggesting osmotically-evoked hypertrophy involves activation of PLC and PKC.

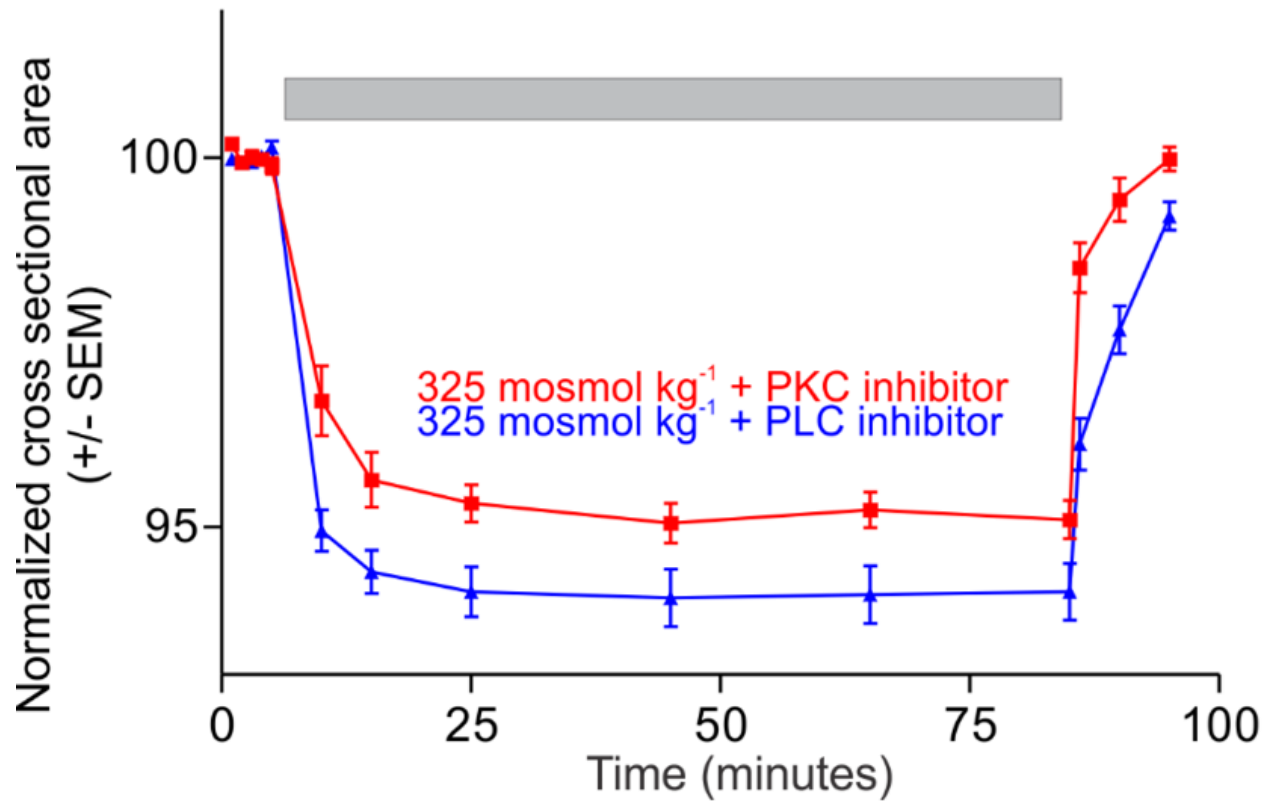


Figure 4.5: Osmotically-evoked hypertrophy involves activation of PLC and PKC. When MNCs are exposed to hypertonic PGC (325 mosmol kg⁻¹) in the presence of a PLC inhibitor (U73122) (n=12) or a PKC inhibitor (bisindolylmaleimide I) (n=12) they show the acute osmotic shrinkage response and remain shrunk over the measured time period. Upon re-exposure to isotonic PGC, they rapidly recover to their control CSA. The period of hypertonic PGC exposure with the enzyme inhibitors is indicated by the grey bar on the top of the plot.

4.5 Hypertrophy can be induced by activating PKC

As shown previously, hypertrophy was prevented in MNCs exposed to a PKC inhibitor. We wanted to test whether activation of PKC is sufficient to cause hypertrophy. Acutely isolated MNCs were exposed to isotonic PGC with a PKC activator (phorbol 12-myristate 13-acetate (PMA); 0.1 μ M). The cells were first incubated in isotonic PGC for 5 minutes followed by addition of isotonic PGC with the PKC activator. The MNCs CSA was monitored at various time points. As shown in Figure 4.6, the MNCs treated with the PKC activator (n=7; 4 experiments) enlarged to a maximum CSA of $102.9 \pm 0.3\%$ of control and remained in the enlarged state, which was statistically different than the mean CSA of the control (using a one-way analysis of variance test; $P < 0.01$). When the PKC activator treated MNCs were re-exposed to normal isotonic PGC, the MNCs rapidly recovered to their respective control sizes and their CSAs were not statistically different than the control CSAs (using a one-way analysis of variance test; $P > 0.05$). A separate experiment was conducted to see if this a true effect of the activator by treating the MNCs with an inactive analogue of the PKC activator (4 β -phorbol 12-myristate 13-acetate; 0.1 μ M). The MNCs treated with the inactive analogue of the PKC activator (n=5; 2 experiments) did not hypertrophy and their mean CSAs were not statistically different at each time point throughout the experiment (using a one-way analysis of variance test; $P > 0.05$). The results suggest that hypertrophy seems to involve activation of PKC as this effect is not seen with the inactive analogue of the PKC activator.

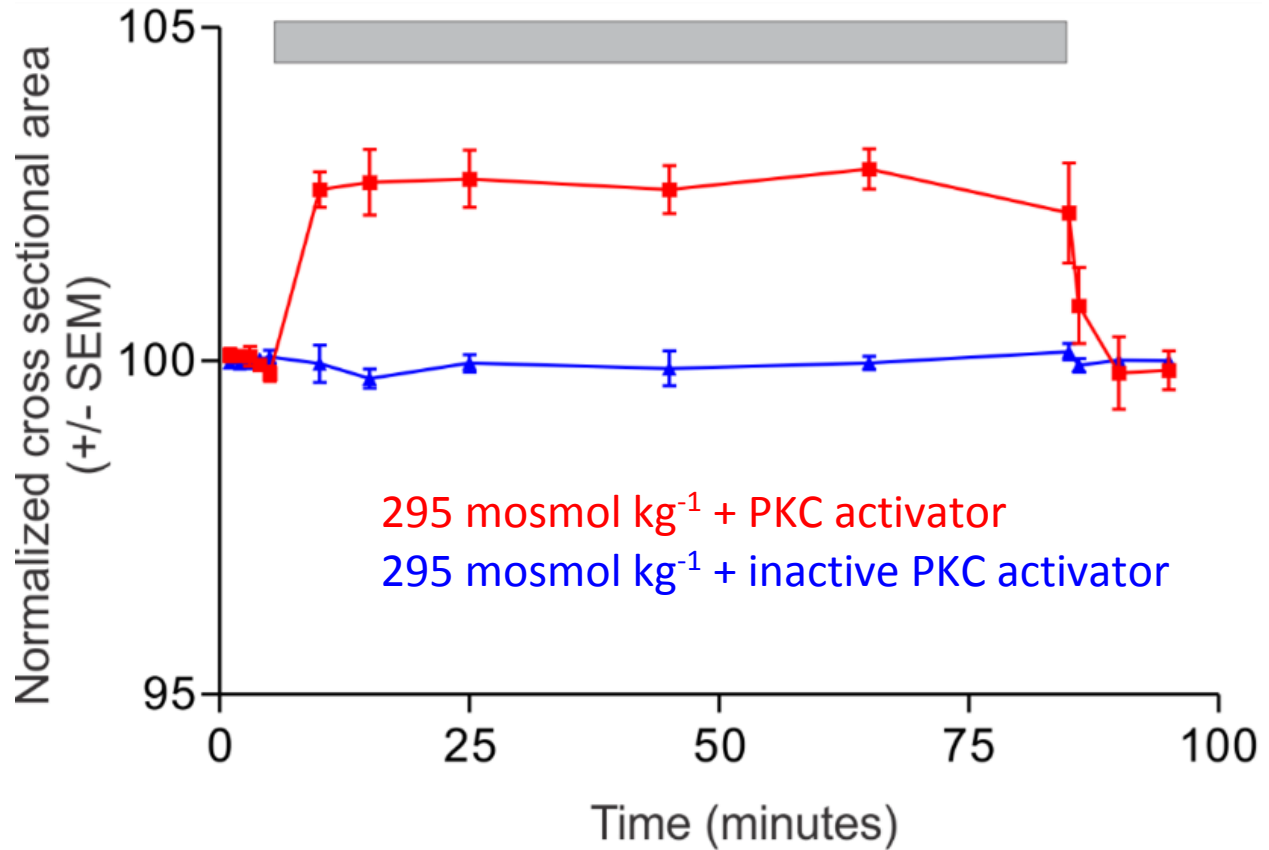


Figure 4.6: Hypertrophy can be induced by activation of PKC. When MNCs are exposed to isotonic PGC with PMA (0.1 μ M) (n=7) they hypertrophy within 5 minutes and remain enlarged over the course of the exposed time period. When MNCs are exposed to isotonic PGC with an inactive analogue of PMA (0.1 μ M) (n=5) they do not show hypertrophy and remain at the control size. Upon re-exposure to normal isotonic PGC, the PMA-treated MNCs rapidly recover to their control CSA. The period of exposure to PMA is indicated by the grey bar on the top of the plot.

4.6 Hypertrophy can be activated by depolarization of the MNCs

Previous lab members have shown that exposing MNCs to TTX in hypertonic PGC prevented hypertrophy, suggesting action potential firing is required for hypertrophy (Shah *et al.*, 2014). We wanted to test whether exposing the MNCs to depolarizing isotonic PGC is sufficient to induce hypertrophy. Acutely isolated MNCs were exposed to isotonic PGC in high $[K^+]$ conditions (25 mM KCl) to depolarize the cells. Using the Goldman-Hodgkin-Katz Equation, it was calculated that the increase in extracellular K^+ results in the MNCs depolarizing to about -40 millivolts (mV), which can cause action potential firing (Boron & Boulpaep, 2008) or open low voltage-gated $Ca_v1.3$ Ca^{2+} channels (Fisher & Bourque, 1995, 1996). The MNCs were first incubated in isotonic PGC for 5 minutes followed by addition of isotonic PGC with high $[K^+]$. The MNCs CSA was monitored at various time points. As shown in Figure 4.7, the MNCs treated with the high K^+ conditions ($n=5$; 2 experiments) enlarged to a maximum CSA of $103.9 \pm 0.4\%$ of control and remained in the enlarged state which was statistically different than the mean CSA of the control (using a one-way analysis of variance test; $P < 0.01$). When the high K^+ treated MNCs were re-exposed to normal isotonic PGC, the MNCs rapidly recovered to their respective control state and their CSAs were not statistically different than the control CSAs (using a one-way analysis of variance test; $P > 0.05$). A separate experiment was conducted to see if this effect can be negated by adding a PLC inhibitor (U73122; 1 μ M). The MNCs treated with high $[K^+]$ isotonic saline with PLC inhibitor ($n=11$; 4 experiments) did not hypertrophy and their mean CSAs were not statistically different at each time point throughout the experiment (using a one-way analysis of variance test; $P > 0.05$). The results suggest hypertrophy requires cell depolarization and this effect can be blocked by inhibiting PLC.

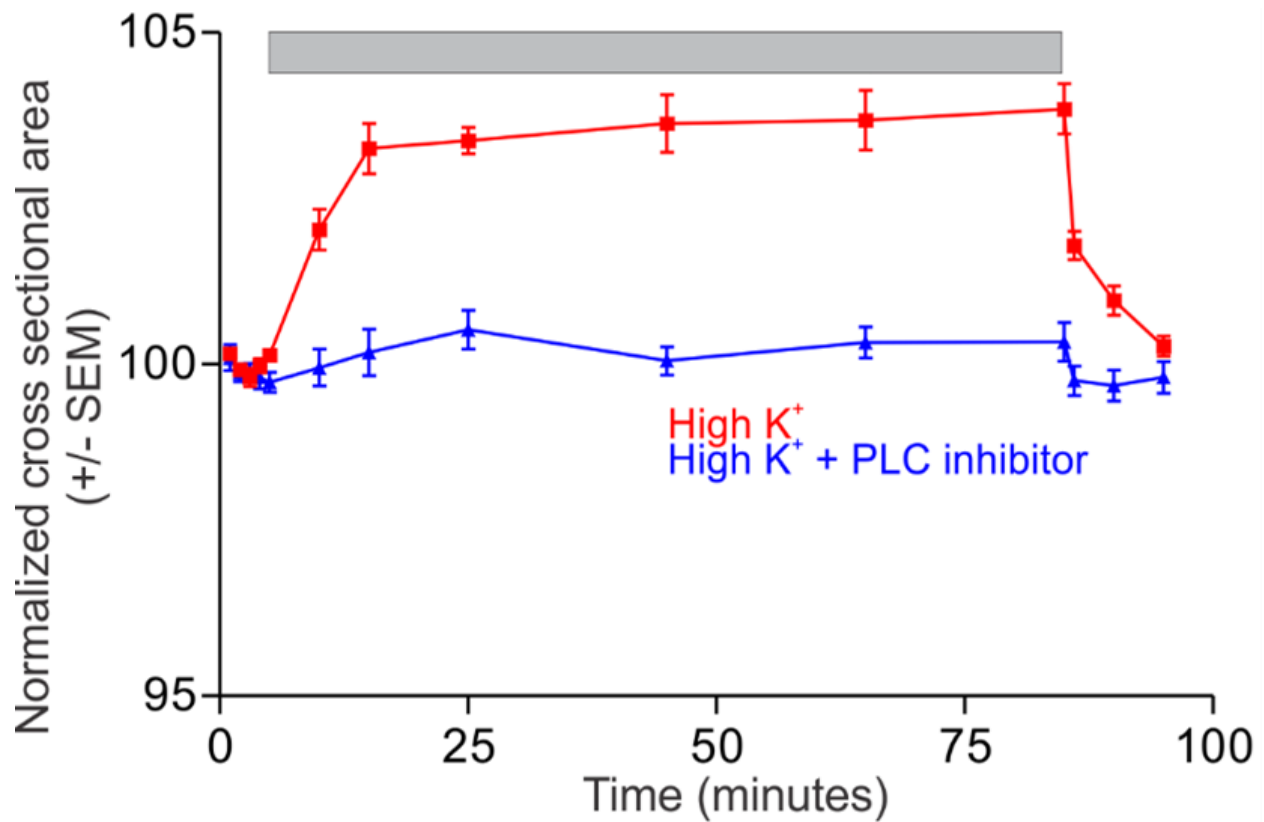


Figure 4.7: Hypertrophy is activated by cell depolarization. When MNCs are exposed to isotonic PGC with high $[K^+]$ (25 mM) (n=5), they hypertrophy within 5 minutes and remain enlarged over the course of the exposed time period. Upon re-exposure to normal isotonic PGC, they rapidly recover to their control CSA. When MNCs are exposed to isotonic PGC with high $[K^+]$ and a PLC inhibitor (n=11), they do not show hypertrophy and remain in the control CSA. The period of exposure to high $[K^+]$ (with or without PLC inhibitor) is indicated by the grey bar on the top of the plot.

4.7 Hypertrophy can be induced by increasing $[Ca^{2+}]_i$

Previous lab members have shown that exposing MNCs to nifedipine or BAPTA-AM in hypertonic PGC, prevented hypertrophy suggesting Ca^{2+} entry is required for hypertrophy (Shah *et al.*, 2014). We wanted to test whether increasing $[Ca^{2+}]_i$ is sufficient to induce hypertrophy. Acutely isolated MNCs were exposed to isotonic PGC with a Ca^{2+} ionophore (A231877; 10 μ M). The cells were first incubated in isotonic PGC for 5 minutes followed by addition of isotonic PGC with the Ca^{2+} ionophore. The MNCs CSA was monitored at various time points. As shown in Figure 4.8, the MNCs treated with Ca^{2+} ionophore (n=9; 4 experiments) enlarged to a maximum CSA of $104.5 \pm 0.4\%$ of control and remained in the enlarged state, which was statistically different than the mean CSA of the control (using a one-way analysis of variance test; $P < 0.01$). When the Ca^{2+} ionophore-treated MNCs were re-exposed to normal isotonic PGC, the MNCs did not recover to their respective control and their CSAs remained statistically different than the control CSAs but not statistically different than the hypertrophied states (using a one-way analysis of variance test; $P < 0.01$ and $P > 0.05$, respectively). The results suggest hypertrophy involves an influx and rise in $[Ca^{2+}]_i$ and recovery seems to involve mechanisms that serves to buffer or extrude the $[Ca^{2+}]_i$.

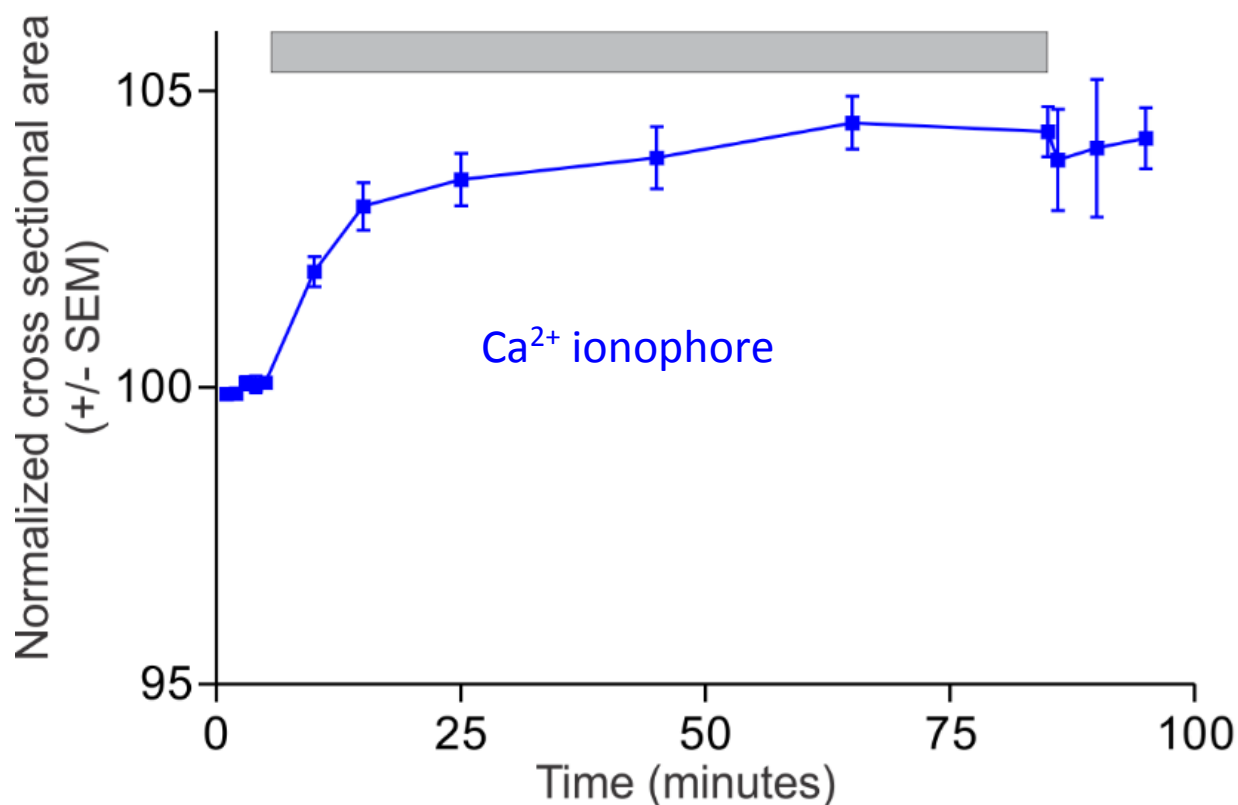


Figure 4.8: Hypertrophy is activated by influx of Ca^{2+} . When MNCs are exposed to isotonic PGC with the Ca^{2+} ionophore, A231877 (10 μM) (n=9) they hypertrophy within 5 minutes and remain enlarged over the course of the exposed time period. Upon re-exposure to normal isotonic PGC, they do not recover to their control CSA during the 10 minutes monitored. The period of exposure to the Ca^{2+} ionophore is indicated by the grey bar on the top of the plot.

4.8 Exposure of MNCs to hypo-osmolar solutions results in a sustained decrease in soma size

A study investigated the cell and nuclear sizes of SON neurons and found that under chronic hypotonic conditions (about 223 mosmol/kg) for 7 days resulted in a 40% decrease in both cell soma and nuclear size (Zhang *et al.*, 2001). We therefore tested whether this *in vivo* finding can be replicated in an *in vitro* model. Acutely isolated MNCs were first incubated in isotonic PGC for 10 minutes followed by switching the stationary bath to 280 mosmol kg⁻¹ hypotonic PGC. The MNCs CSA was monitored at various time points. The MNCs exposed to hypotonic PGC (n=9; 1 experiment – 2 frames of view) enlarged to a maximum CSA of $103.3 \pm 0.3\%$ of control after 10 minutes and shrunk to a minimum CSA of $97.6 \pm 0.3\%$ after 80 minutes. The mean enlarged and shrunken CSA of hypotonic PGC treated cells measured at (15 and 85 minutes, respectively), were significantly different than the mean CSA of the control (using a one-way repeated measures analysis of variance test; $P < 0.01$ in both cases). The mean CSA of the enlarged and shrunken states of the hypotonic PGC treated MNCs were statistically different than the mean CSA of MNCs treated with isotonic PGC at the same time periods (using a two-way analysis of variance; $P < 0.01$ in both cases). When the hypotonic PGC-treated MNCs were returned to the isotonic PGC, they rapidly returned to their control CSA after about 5-10 minutes. The results are shown below in Figure 4.9 and show that opposite to hypertonic-induced hypertrophy, MNCs can undergo the reverse, hypotonic-induced decrease in cell size.

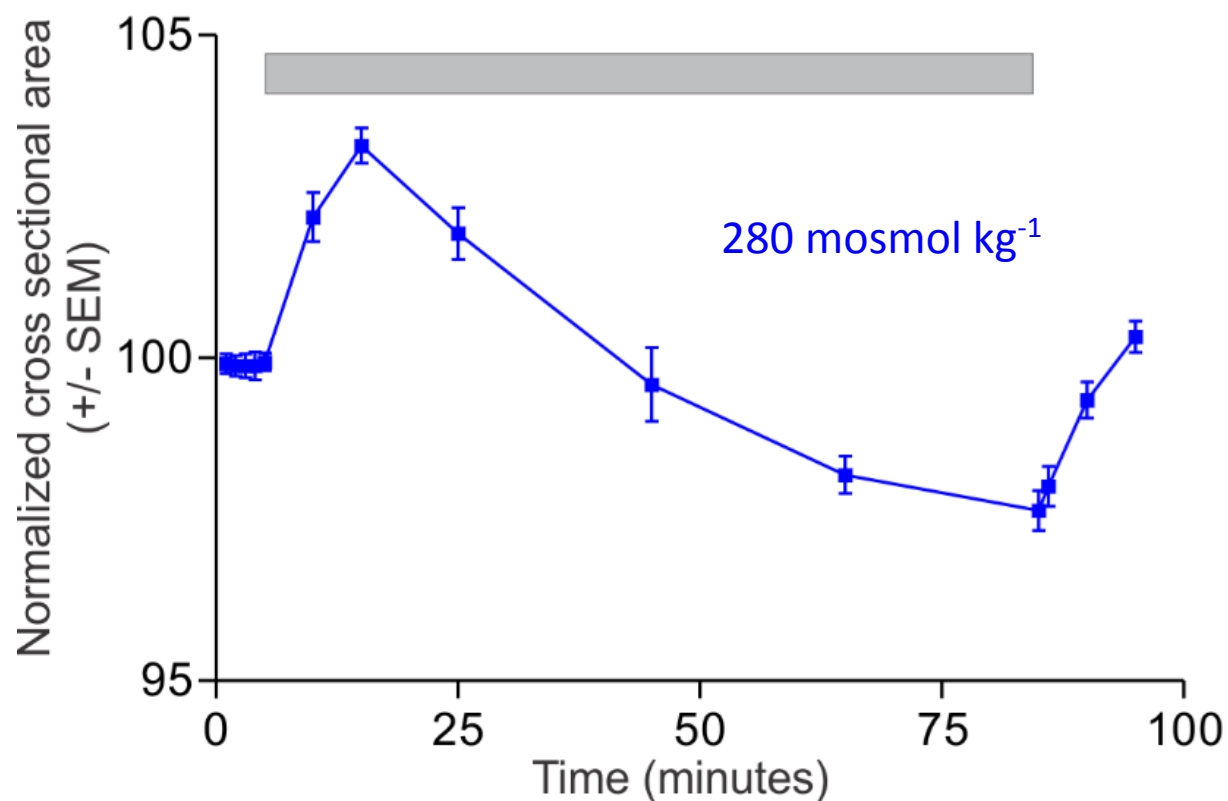


Figure 4.9: Hypo-osmolality evokes a reversible decrease in MNC size. Exposure to hypotonic PGC (n=9) causes acutely isolated MNCs to enlarge and shrink over 80 minutes. Returning the MNCs to isotonic PGC results in a rapid reversal of hypotrophy to the control CSA. The period of hypotonic PGC exposure is indicated by the grey bar at the top of the plot.

4.9 Immunofluorescence of L-type Ca^{2+} subunit $\text{Cav}1.2$ in MNCs, increases after hypertonic exposure

It is known that chronically dehydrated rats have increased L-type Ca^{2+} current density (Zhang *et al.*, 2007a) and the $\text{Cav}1.2$ subunit is expressed in MNC soma and dendrites (Glasgow *et al.*, 1999; Joux *et al.*, 2001). We therefore employed immunocytochemical techniques to measure the density of $\text{Cav}1.2$ subunits in MNCs after exposure to hypertonic PGC. Acutely isolated MNCs were treated with isotonic PGC, hypertonic PGC ($325 \text{ mosmol kg}^{-1}$) or hypertonic PGC with TTX for 120 minutes. A protocol for immunolabeling fixed MNCs with antibodies against $\text{Cav}1.2$ was used to quantify the difference in expression between the treatment groups. The qualitative pictures of MNCs stained in different treatments is presented below in Figure 4.10 showing the MNCs with diffuse staining of $\text{Cav}1.2$ subunits on the cell plasma membrane and cytosol. The MNCs treated with hyperosmolar treatment showed higher immunostaining than the MNCs treated with isotonic solution. As shown in Figure 4.11, the mean fluorescence intensity of hypertonic PGC treated MNCs was $37.2 \pm 15.6\%$ ($n=64$) higher than the MNCs treated with isotonic PGC ($n=68$) (62.4 ± 6.2 to 85.6 ± 9.7 arbitrary units/ μm^2) and was statistically different (using a one-way repeated measures analysis of variance test; $P < 0.05$). The mean fluorescence intensity of hypertonic PGC with TTX treated MNCs was $28.4 \pm 12.2\%$ ($n=70$) higher than the MNCs treated with isotonic PGC ($n=68$) (62.4 ± 6.2 to 80.1 ± 7.6 arbitrary units/ μm^2) and was statistically different (using a one-way repeated measures analysis of variance test; $P < 0.05$). The mean fluorescence intensities of hypertonic PGC treated MNCs with and without TTX were not significantly different (using a one-way repeated measures analysis of variance test; $P > 0.05$). The results suggest that there is an increase in $\text{Cav}1.2$ expression in MNCs exposed to hypertonic PGC, which does not depend on action potential firing.

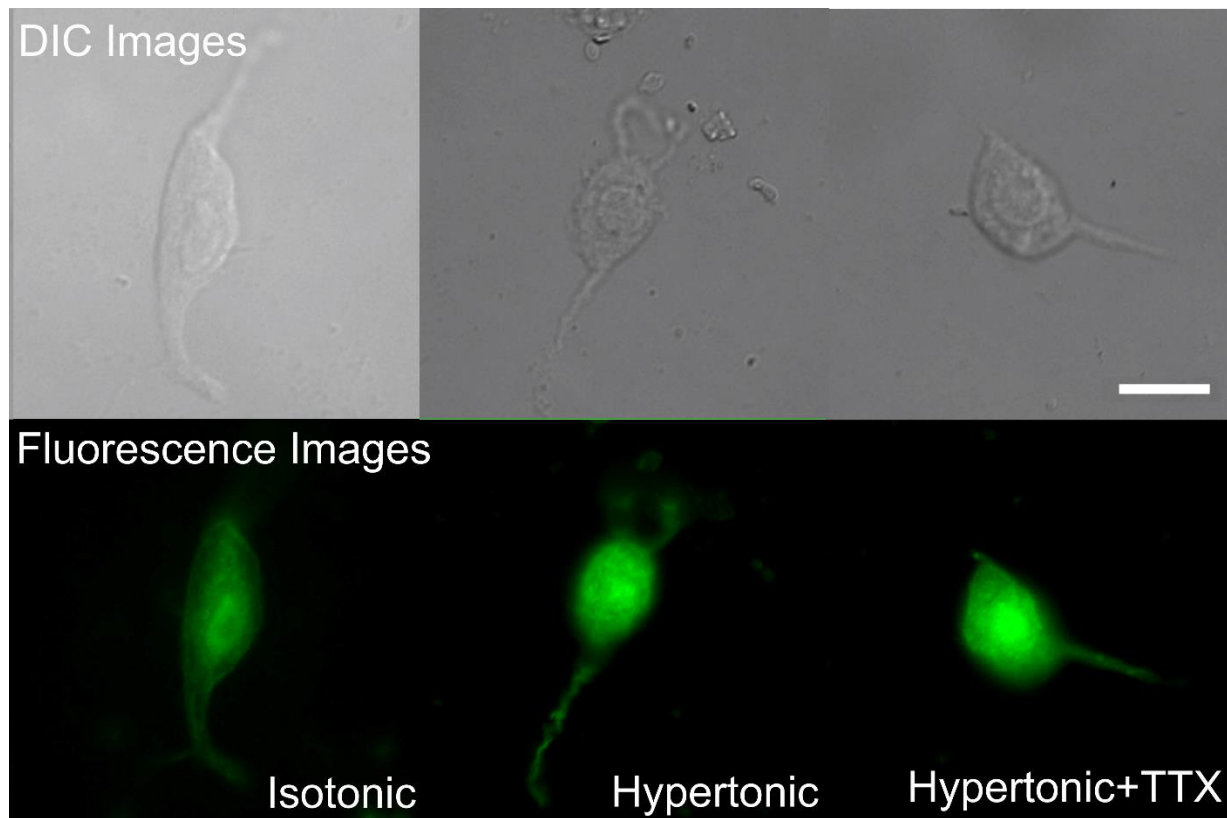


Figure 4.10: Pictures of MNCs showing increased immunofluorescence of the Cav1.2 subunit after hypertonic exposure. Representative MNCs exposed to different treatments are shown as DIC images and fluorescent images. The top row shows the MNCs through a DIC filter while the bottom row green colour represents MNC immunostaining with a Cav_v1.2 subunit antibody. The scale bar represents a length of 25 μ m.

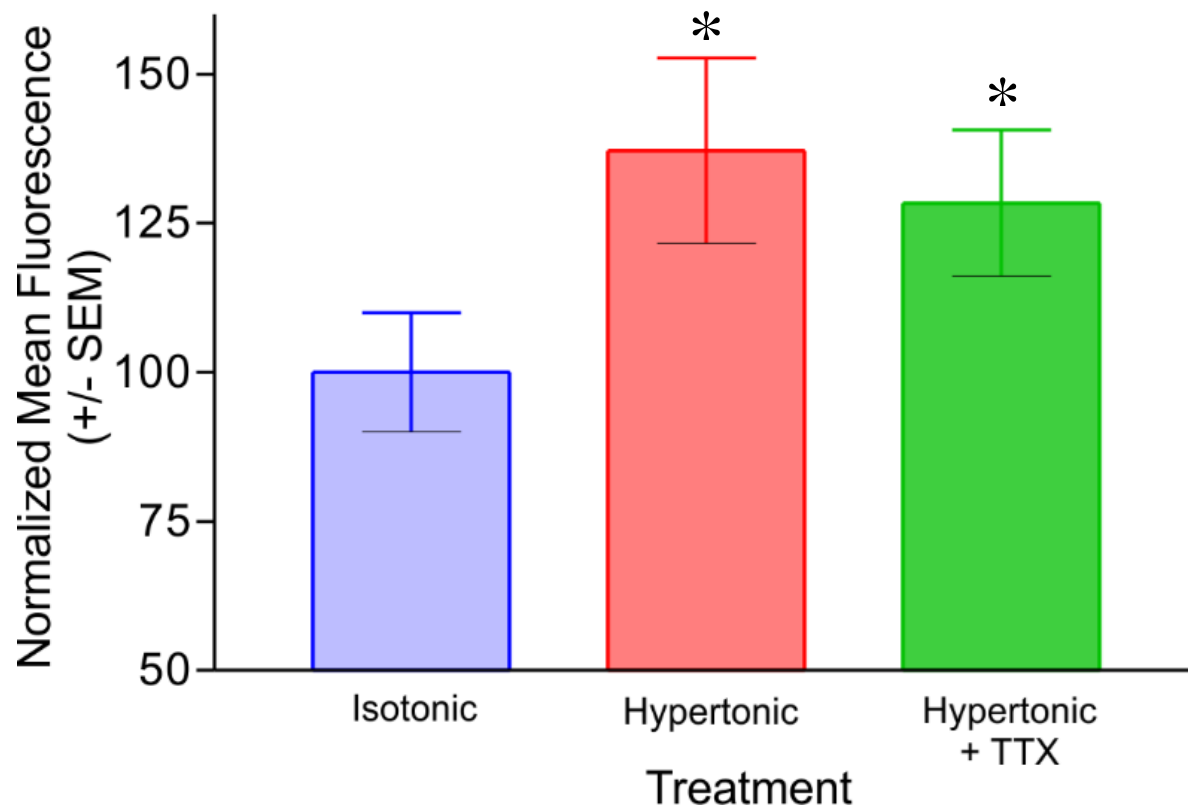


Figure 4.11: Bar graph of MNCs showing increased immunofluorescence of the Cav1.2 subunit after hypertonic exposure. The bar graphs show the normalized mean fluorescence to Cav1.2 antibody in MNCs exposed to different treatments. Data are expressed as mean normalized fluorescence \pm SEM. Hypertonic exposure causes a significant increase in Cav1.2 expression compared to isotonic treated MNCs. The addition of TTX to hypertonic exposure does not statistically affect the expression of the Cav1.2 subunit. The asterisks represent a statistical significance of $P \leq 0.05$ compared to isotonic controls.

CHAPTER 5: DISCUSSION

The structural adaptations of MNCs under chronic osmotic stress is poorly understood, due in part to the difficulty in elucidating the mechanisms in an *in vivo* preparation. Our lab has recently established an *in vitro* preparation using acutely isolated MNCs, which was used to pharmacologically assess the osmotically-evoked hypertrophy (Shah *et al.*, 2014). MNCs were exposed to sustained increases in osmolality and it was observed that in addition to the acute shrinkage, MNCs can hypertrophy over the course of tens of minutes (Shah *et al.*, 2014). This effect was dose-dependent, as decreases in the osmolality of the hypertonic solution results in comparable decreases in shrinkage and hypertrophy. Exposing the MNCs to hypotonic solution resulted in an immediate enlargement followed by a sustained decrease in cell size. It was also concluded that recovery from hypertrophy requires dynamin-dependent endocytosis. Further experimentation revealed that osmotically-evoked hypertrophy is independent of NKCC1 activation (involved in cell volume regulatory processes), requires activation of PLC, PKC, cell depolarization and increases in $[Ca^{2+}]_i$. An *in vitro* preparation of acutely isolated MNCs exposed to hypertonic conditions for 120 minutes has shown increased immunolabeling of the L-type Ca^{2+} channel subunit, $Ca_v1.2$, which could be the result of increased channel subunit synthesis. This chapter will further elaborate on the findings revealed in this thesis and will attempt to draw physiological implications to the roles of these adaptations in neuroendocrine cells.

5.1 Osmotically-induced structural changes

During acute phases of hypertonic or hypotonic stress, the MNCs, like most other cell types, undergo acute cell volume shrinkage and enlargement, respectively (Lang *et al.*, 1998a; Lang *et*

al., 1998b; Zhang & Bourque, 2003; Lang, 2007). The MNCs only return to their original cell volume, when re-exposed to isotonic conditions (Zhang & Bourque, 2003). This is in contrast to other neuronal cell types such as hippocampal neurons which undergo a dynamic RVI or RVD in order to compensate for the changes evoked by acute osmotic stress (Lang *et al.*, 1998a; Lang *et al.*, 1998b; Zhang & Bourque, 2003). In MNCs, the lack of compensatory volume regulatory processes affords the cell the ability to transduce changes in ECF osmolality into changes in membrane potential (Zhang & Bourque, 2003). The cells of the SON, MNCs and astrocytes, are also able to structurally adapt to sustained hypertonic exposure (Hatton, 1997). Chronic dehydration caused by salinized drinking water results in a 170% increase in MNC surface area in electron micrographs (Modney & Hatton, 1989). Chronic hypotonic conditions have been shown to display the opposite effect including sustained decreases (~40%) in cellular and nuclear size (Zhang *et al.*, 2001). These changes in the structure of the MNCs and ultimately the SON are thought to occur to ensure long-term hormonal output during times of chronic osmotic stress (Hatton, 1997).

5.1.1 Osmotically-evoked hypertrophy occurs *in vitro* and depends on specific cellular processes

Data from previous lab members suggests that hypertrophy is dependent on specific cellular activities (Shah *et al.*, 2014). The preincubation of MNCs with several blockers such as TTX, SB366791 and nifedipine in hypertonic solution resulted in shrinkage and recovery but no hypertrophy (Shah *et al.*, 2014). This suggests that hypertrophy requires action potential firing, activation of the SIC channel, and influx of Ca^{2+} through L-type Ca^{2+} channels (Shah *et al.*, 2014). Furthermore, adding these blockers to hypertrophied cells resulted in rapid reversal of the

hypertrophy, suggesting MNCs must maintain these processes to maintain hypertrophy (Shah *et al.*, 2014). Finally, in an effort to understand the mechanism of enlargement, the role of exocytosis was further probed by incubating the MNCs in hypertonic solution with a peptide, TAT-NSF700, which is an inhibitor of SNARE-mediated exocytotic fusion (Shah *et al.*, 2014). It was noted that the TAT-NSF700 peptide prevented hypertrophy, suggesting that the cell enlargement in hypertrophy is due to insertion of intracellular membrane (Shah *et al.*, 2014). This finding was confirmed electrophysiologically by showing that the capacitance of MNCs treated with hypertonic PGC for 2 hours was significantly higher than the capacitance of MNCs in isotonic PGC (Shah *et al.*, 2014).

As stated, the SIC activation and generation of action potentials is necessary for the cells to initiate and maintain hypertrophy (Shah *et al.*, 2014). Thus, even though the MNCs hypertrophy, the SIC is still able to remain activated (Shah *et al.*, 2014). A possible explanation for this is that the SICs are remaining active by some other mechanism. This mechanism may include mediators which serve to directly activate the SICs or may involve the reduction of the inhibitory influence of channel modulating agents, possibly PIP₂ (Brauchi *et al.*, 2007; Rohacs *et al.*, 2008; Ufret-Vincenty *et al.*, 2011). In addition, during membrane insertion while the MNCs hypertrophy, there may be translocation of SICs which may lead to an increase in SIC currents (Morenilla-Palao *et al.*, 2004). This sustained activation of the SIC can explain the depolarization required to facilitate action potential firing, even in the hypertrophied state.

It is interesting to note that the *in vivo* studies show that MNC hypertrophy can occur up to about 170% (Modney & Hatton, 1989) in MNC size, however this *in vitro* preparation shows only a 5%

increase in CSA. This discrepancy can be due to many reasons such as time of exposure and also by the absence of surrounding astrocytes in the acutely isolated preparation, which may release factors involved in hypertrophy (Shah *et al.*, 2014). For example, it is known that the astrocytes surrounding MNCs release ATP which can act on purinergic P2X₇ receptors on MNCs to increase excitatory postsynaptic potentials (Gordon *et al.*, 2009). Thus the presence of astrocytes may enhance the hypertrophy response as seen *in vivo* via the release of ATP. In addition, the *in vivo* preparation consists of closely packed MNCs and so somatodendritic release of certain autocrine or paracrine mediators such as dynorphin (Whitnall *et al.*, 1983; Simmons *et al.*, 1995) and VP (Ludwig & Leng, 1997; Ludwig, 1998b) could also enhance the hypertrophy response.

In addition, capacitance measurements measured in the lab show an increase of 7% in hypertonic conditions (325 mosmol kg⁻¹), however there is an 11% increase from the shrunken CSA to the hypertrophied CSA (Shah *et al.*, 2014). The lower increase in capacitance may reflect the fact that whole-cell capacitance represents total membrane surface area including the MNC soma and dendrites (Shah *et al.*, 2014). With this rationale, increasing the cell soma size by a certain amount would therefore result in a lower increase in total membrane surface area and thus whole-cell capacitance (Shah *et al.*, 2014). Finally, the MNCs contain processes and are not perfect spheres thus increases in CSA may not accurately translate into increases in total membrane surface area (whole-cell capacitance).

The results discussed were obtained previously by other lab members. I will now discuss the results that I obtained.

5.1.1.1 Hypertrophy of MNCs is dose-dependent

When MNCs were treated with 325 mosmol kg⁻¹ hypertonic PGC, they immediately shrunk on average to about 94.5% of their control CSA and maintained their volume for about 20 minutes, after which they started to hypertrophy. This finding is agreement with studies showing that MNCs shrink immediately and remain shrunk over the course of 10 minutes, suggesting they do not undergo acute cell volume regulation (Zhang & Bourque, 2003). The increase in osmolality was sustained and it was observed that over the course of 90 minutes the MNC cell soma increases to about 105.2%. This observation is also in agreement with studies showing dehydration lasting as little as 2 hours can induce hypertrophy in the MNC soma *in vivo* (Hatton & Walters, 1973). Upon re-introducing isotonic PGC, the MNCs rapidly recovered to the control CSA, a finding that is in agreement with the reversibility of MNC soma hypertrophy (Hatton, 1997). An interesting finding within this study is that hypertrophy occurs at more physiological increases in ECF osmolality as evident by a reduced shrinkage and hypertrophy in MNCs exposed to 305 and 300 mosmol kg⁻¹ hypertonic PGC. This suggests that hypertrophy occurs at physiological ranges and that the response is sensitive to even small changes in osmolality. This finding suggests that osmotically-evoked hypertrophy may occur on a day-to-day basis. This dynamic regulation of MNC size and perhaps excitability may regulate the VP output and overall fluid balance.

5.1.1.2 Hypertrophy does not involve NKCC1-mediated cell volume regulation

Osmosis dictates that water will only flow from hypotonic to relatively hypertonic compartments to equalize the osmolality (Boron & Boulpaep, 2008). Under hypertonic conditions, the MNCs hypertrophy, which is known to require SNARE-mediated exocytotic fusion of membrane (Shah

et al., 2014). Despite the addition of membrane, the increase in cell volume requires regulation of cytoplasmic osmotic strength to enable the increase in intracellular water content (Lang *et al.*, 1998a; Lang *et al.*, 1998b; Lang, 2007; Shah *et al.*, 2014). Thus the cell must ultimately increase the osmotic strength of its cytoplasm by increasing the concentration of ions and organic molecules (Lang *et al.*, 1998a; Lang *et al.*, 1998b; Lang, 2007). It was therefore important to address this issue to see if ion transporters involved in RVI could be involved in hypertrophy. NKCC1, a $\text{Na}^+/\text{K}^+/\text{Cl}^-$ co-transporter, is a major accumulator of ions in RVI (Lang *et al.*, 1998a; Lang *et al.*, 1998b). When MNCs were exposed to hypertonic PGC in the presence of bumetanide, there was no significant difference in CSAs when compared to MNCs exposed to hypertonicity without bumetanide. This effect was important since NKCC1 is involved in RVI, and thus these findings suggest that if hypertrophy involves sequestration of ions in the cytosol, then it is not through activation of NKCC1. Since hypertrophy involves insertion of membrane at the surface, there could be translocation of certain ionic and organic molecule transporters to increase the intracellular osmotic strength and water content resulting in cell volume expansion. Further studies in this *in vitro* preparation need to carefully examine the role of other ionic transporters in hypertrophy. In addition, it is known that chronic hypertonic stress causes upregulation of several genes (Glasgow *et al.*, 2000; Burbach *et al.*, 2001; Yue *et al.*, 2006). It is thus possible that an increase in intracellular osmolality may be provided, in part, by an increase in gene expression. Our studies on the effect of hypertonicity on L-type Ca^{2+} channels have shown that a short two hour exposure is sufficient to cause a significant increase in the synthesis of the $\text{Ca}_v1.2$ subunit. Thus other genes controlled by similar gene upregulation pathways can also be rapidly increased and contribute to the increase in intracellular osmolality. Further, endogenous synthesis of other types of biomolecules could also contribute to an increase in intracellular osmolality. Additional

experiments should be performed to understand whether rapid synthesis of proteins and other biomolecules are important in contributing to the increase in intracellular osmolality and MNC hypertrophy.

5.1.1.3 Dynamin-dependent endocytosis governs the MNC recovery from hypertrophy

The hypertrophy of MNCs occurs through exocytotic fusion of intracellular membranes as confirmed by pharmacological and electrophysiological observations in our preparation (Shah *et al.*, 2014). During acute hypertonic stress, the MNCs shrink in size as water rushes out of the AQP channels to equalize the osmolality (Zhang & Bourque, 2003; Boron & Boulpaep, 2008). Once the MNCs undergo exocytotic increases in membrane and hypertrophy, there must be a mechanism to revert back to the normal isotonic CSA. Many neuronal cells exhibit reversible structural and functional adaptations with exocytosis and endocytosis acting as a mechanistic “switch”. An example is in long-term potentiation (LTP) during which AMPARs are translocated in the postsynaptic membrane to sustain excitatory post synaptic potentials (Lu *et al.*, 2001). During long-term depression (LTD), the AMPARs are not required and thus the neuron activates dynamin-dependent endocytotic machinery to reduce their presence on the membrane (Beattie *et al.*, 2000). A similar process may exist within the MNCs as hypertrophy may be analogous to LTP (increased channels and receptors) to sustain high amounts of hormonal output, while recovery from hypertrophy may be analogous to LTD by withdrawing membrane (including receptors and channels) and thus decreasing neuronal volume. In order to test this hypothesis, a blocker of dynamin-dependent endocytosis, dynasore, was employed to study recovery from MNC hypertrophy (Macia *et al.*, 2006; Kirchhausen *et al.*, 2008). Dynamin is a GTPase that is the final requirement to pinch off the invaginations to form intracellular vesicles (Morlot & Roux, 2013).

It was observed that MNCs exposed to hypertonic PGC with dynasore did not differ in any parameter of the hypertrophy (time course, maximal CSA) but failed to recover after re-exposure to isotonic PGC. This suggests that recovery from hypertrophy is a plasma membrane trafficking event that involves dynamin-dependent endocytosis to decrease the MNC CSA. An interesting feature of the recovery in dynasore-treated MNCs is the rapid transient decrease in cell size, followed by a gradual return to the hypertrophied cell size. This is very similar, in non-dynasore-treated MNCs, to the rapid decrease and overshoot in cell size, followed by a steady return to the control size. We do not understand these observations and future studies should aim to resolve these idiosyncrasies in the recovery response.

5.1.1.4 Activation of the PLC-PKC pathway is required for MNC hypertrophy

Hypertonic stress activates a wide variety of protein kinases and phospholipases in non-neuronal cells (Munnik *et al.*, 2000b; Droillard *et al.*, 2002; Rodriguez *et al.*, 2002; Fujii & Zhu, 2012). The phospholipid PIP₂ is a very important modulator of several ion channels especially TRP channels and M-type K⁺ channels (Hilgemann *et al.*, 2001; Suh & Hille, 2005). It has been recently demonstrated that acute hypertonic stress activates PLC in MNCs, resulting in a ~30% decrease in surface PIP₂ levels (Shah *et al.*, 2014). PKC is involved in translocation of TRPV1 in oocytes (Morenilla-Palao *et al.*, 2004) and Ca²⁺ channels in *Aplysia* bag cell neurons (Strong *et al.*, 1987). Further studies have shown translocation of several channels and receptors in MNCs during chronic hyperosmotic stress, including Na⁺ channels (Tanaka *et al.*, 1999), V_{1a} receptors (Hurbin *et al.*, 2002), dynorphin receptors (Shuster *et al.*, 1999) and L-type Ca²⁺ channels (Zhang *et al.*, 2007a). These data warrant further investigation in MNCs for the role of PLC and PKC activation in triggering hypertrophy. It was observed that MNCs treated with hypertonic PGC in the presence

of an inhibitor of either PLC or PKC, did not hypertrophy, suggesting their activation is required for the hypertrophic response. Further, it was shown that treating the MNCs with a PKC activator in the presence of isotonic PGC resulted in a mild hypertrophic response. The smaller size of the PKC activator-induced hypertrophy suggests that other triggers are required for the full hypertrophic response such as increased $[Ca^{2+}]_i$. Since the MNC osmosensitivity requires TRPV1 channels (Zhang *et al.*, 2007b; Sharif-Naeini *et al.*, 2008) and also since Ca^{2+} channels are found in intracellular granules of MNCs (Fisher *et al.*, 2000), it is possible that hypertrophy leads to translocation of these channels resulting in hypertrophy and modulation of MNC electrophysiology.

5.1.1.5 Cell depolarization and Ca^{2+} influx can cause MNC hypertrophy

Previous data from the lab suggested that MNC hypertrophy requires action potential firing and Ca^{2+} influx through L-type Ca^{2+} channels for the initiation and maintenance of hypertrophy (Shah *et al.*, 2014). The ability of cell depolarization and influx of Ca^{2+} through an ionophore was further investigated to establish whether these events are sufficient to trigger hypertrophy in the absence of an increase in osmolality. To probe the effects of cell depolarization, an isotonic PGC containing high K^+ (25 mM) was used to cause cell depolarization. It was shown that MNCs treated with a high K^+ isotonic PGC rapidly hypertrophied and recovered when they were reintroduced to normokalemic isotonic PGC. Furthermore, this effect was negated by the addition of a PLC inhibitor to the high K^+ isotonic PGC, suggesting PLC is activated downstream of cell depolarization and is required for the depolarization to cause an hypertrophy. Secondly, a Ca^{2+} ionophore, A23187, was used to rapidly increase $[Ca^{2+}]_i$ and probe whether it is sufficient to cause hypertrophy. MNCs exposed to the Ca^{2+} ionophore in isotonic PGC showed a rapid hypertrophy but were not able to recover when re-exposed to isotonic PGC without the Ca^{2+} ionophore. The

inability to recover from hypertrophy after addition of the Ca^{2+} ionophore is expected since the ionophore cannot be removed from the membrane (Herth, 1978; Hadley, 1987) probably due to its lipophilic nature (Reed & Lardy, 1972; Klausner *et al.*, 1979). It would also be useful to see if treatment with EGTA or BAPTA-AM to buffer internal Ca^{2+} could prevent hypertrophy. In addition, to further confirm that the increase in $[\text{Ca}^{2+}]_i$ is indeed the trigger (and not cell damage), the Ca^{2+} ionophore maybe co-administered with an inhibitor of PLC or PKC. These data suggest that hypertonicity-induced hypertrophy requires cell depolarization-induced Ca^{2+} influx, and the activation of PLC and PKC, and exocytotic fusion.

5.1.1.6 Hypotonicity evokes reversible hypotrophy in MNCs

A study documenting the effects of long-term *in vivo* hypotonicity in MNCs showed that MNC soma and nuclear sizes decreased 40% in size (Zhang *et al.*, 2001). This effect is opposite to what is seen during chronic hypertonic conditions and is interesting that MNCs are able to adapt to a wide variety of osmotic stresses in opposite ways. This finding is still poorly studied and the mechanisms underlying the decrease in cell size should be further investigated. The acutely isolated MNC preparation was used to explore whether the *in vivo* results can be replicated *in vitro*. The MNCs were exposed to hypotonic PGC (280 mosmol kg^{-1}) and showed immediate cellular enlargement, as expected (Zhang *et al.*, 2001). During a course of 75 minutes, however, the MNCs gradually decreased in cell size. The decrease in the MNCs cell size was sustained, but they recovered to their control CSA upon returning to isotonic conditions. Thus the *in vivo* results could be replicated and the *in vitro* preparation can be further used to describe the mechanism of this hypotonic-evoked decrease in cell size.

5.2 Osmotically-induced functional changes

The structural adaptations described above are part of the functional adaptations that help to modulate the electrical firing pattern and hormonal output (Hatton, 1997). These changes include upregulation of specific ion channels and receptors on the cell membrane and expression of genes. The V_{1a} receptor is upregulated at the MNC plasma membrane and it serves to detect the increased VP output and inhibit phasic firing in MNCs (Petersen, 2006). Na^+ channels (Tanaka *et al.*, 1999) are also translocated to the cell surface and this may alter the electrophysiological properties and phasic firing patterns. Translocation of dynorphin receptors occurs and their role involves decreasing Ca^{2+} influx and thus decreasing neuropeptide release (Shuster *et al.*, 1999; Brown & Bourque, 2004, 2006). In terms of genes, several genes involved in general cell metabolism, housekeeping and cytoarchitecture are upregulated in order to prepare the MNC for sustained output of hormones (Glasgow *et al.*, 2000; Burbach *et al.*, 2001; Yue *et al.*, 2006).

5.2.1 Immunostaining of Cav1.2 L-type Ca^{2+} channel subunit increases in MNCs exposed to sustained hypertonicity

L-type Ca^{2+} channels with subunits Cav1.2 and Cav1.3 have been identified in MNCs (Glasgow *et al.*, 1999; Joux *et al.*, 2001). These L-type Ca^{2+} channels are found on the membranes of the soma and dendrites (Fisher & Bourque, 1995; Joux *et al.*, 2001; Zhang *et al.*, 2007a) and there also exists an internal pool of VGCCs that may include L-type Ca^{2+} channels (Fisher *et al.*, 2000). A study from our lab has demonstrated that rats dehydrated overnight show an 80% increase in L-type Ca^{2+} currents using whole-cell patch clamp (Zhang *et al.*, 2007a). It is also known that Ca^{2+} currents do not increase immediately following exposure to hypertonic solutions (Liu *et al.*, 2005). The results from this study document that exposing MNCs to hypertonic solution for 2 hours

causes an increase in Cav1.2 immunostaining of about 37%. This increase was not blocked by addition of TTX suggesting that action potential firing is not required for the increase in the Cav1.2 subunit.

The increase in Cav1.2 immunolabeling suggests an increase in channel number, however, microarray analysis, did not show an increase in any Ca²⁺ channel mRNA following dehydration (Hindmarch *et al.*, 2006) and RT-PCR did not show an increase in mRNA of L-type Ca²⁺ channels (Zhang *et al.*, 2007a). Thus a possible explanation could be that there is an increase in protein translation as the mammalian target of rapamycin (mTOR), a regulator of protein synthesis (Hay & Sonenberg, 2004), is shown to be activated by hyperosmotic stress in HEK293 cells (Kwak *et al.*, 2012). In our *in vitro* immunocytochemical study, translocation was difficult to examine due to the inability to discern cytoplasmic immunolabeling from plasma membrane immunolabeling as the staining was diffuse. VGCCs are expressed in intracellular vesicles in MNCs and translocation of these channel reserves could be possible (Fisher *et al.*, 2000).

5.3 Physiological consequences of MNC hypertrophy and increases in Cav1.2 subunit

The sustained dehydration in rats results in increases in MNC soma size *in vivo* (Hatton & Walters, 1973), and increases in channels and receptors on the plasma membrane (Shuster *et al.*, 1999; Tanaka *et al.*, 1999; Hurbin *et al.*, 2002; Zhang *et al.*, 2007a). Using the *in vitro* preparation, we may be finally beginning to understand the mechanisms of MNC hypertrophy (Hatton, 1997). Based on our findings in the lab, we can offer a preliminary model of the hypertonicity-induced MNC hypertrophy. During the initial phases of hypertonic exposure, MNCs will undergo immediate shrinkage, which is proportional to the increase in osmolality of the hypertonic solution

(Lang *et al.*, 1998a; Lang *et al.*, 1998b; Zhang & Bourque, 2003). The initial shrinkage will result in activation of SICs, which will allow influx of cations into the MNC and cause depolarization, and facilitate the generation of action potentials (Zhang *et al.*, 2007b; Sharif-Naeini *et al.*, 2008). The action potentials will cause high threshold L-type Ca^{2+} channels (Simmons *et al.*, 1995; Lipscombe *et al.*, 2004; Zhang *et al.*, 2007a) to open and cause an influx and rise in $[\text{Ca}^{2+}]_i$. The rise in $[\text{Ca}^{2+}]_i$ will lead to activation of PLC, which will cleave PIP_2 into IP_3 and DAG (Rebecchi & Pentyala, 2000; Irino *et al.*, 2004). The IP_3 may act to mobilize Ca^{2+} from intracellular stores in the ER (Rebecchi & Pentyala, 2000; Irino *et al.*, 2004). The DAG will bind to and activate PKC, which has been implicated in exocytosis and translocation of many channels and receptors (Morenilla-Palao *et al.*, 2004; Morgan *et al.*, 2005).

From our data it is known that osmotic activation of PLC results in PKC activation. The activation of PKC has been widely implicated in the fusion of vesicles (Naor *et al.*, 1989; Morgan & Burgoyne, 1992; Morgan *et al.*, 2005) and translocation of several channels and receptors (Strong *et al.*, 1987; Morenilla-Palao *et al.*, 2004). In addition, several studies in MNCs have shown that chronic hypertonicity involves upregulation of several channels and receptors including V_{1a} receptors (Hurbin *et al.*, 2002), Na^+ channels (Tanaka *et al.*, 1999) and dynorphin receptors (Shuster *et al.*, 1999) which are all involved in the MNC electrophysiology and hormonal output (Ludwig, 1998b; Brown & Bourque, 2004; Brown *et al.*, 2004). Thus these findings lead to the possibility that the osmotically-evoked hypertrophy, via PKC-mediated fusion of intracellular membranes, can cause translocation of certain channels and receptors to modulate the activity of MNCs and allow functional adaptation for long-term hormonal output. Finally during recovery,

there seems to be an activation of dynamin-dependent endocytotic mechanisms, which serves to rapidly retract the membrane resulting in the recovery of MNC cell size.

The L-type Ca^{2+} channels have a prominent role in MNC neuropeptide secretion and possibly gene transcription (Dolmetsch *et al.*, 2001a), and so can be part of functional adaptations to chronic hypertonicity (Fisher & Bourque, 1995, 1996; Fisher *et al.*, 2000; Fisher & Bourque, 2001). It is well known that Ca^{2+} influx through L-type Ca^{2+} channels can activate CREB and increase transcription of various genes (Bading *et al.*, 1993; Ginty *et al.*, 1993) and this could be part of the long-term response to chronic hypertonicity. The L-type Ca^{2+} channels possess two very important sequences that afford them the ability to activate CREB. The first sequence is the in the C-terminus of Cav1.2 and Cav1.3 subunits, which interacts with anchoring proteins to position them so that they activate CREB (Dolmetsch *et al.*, 2001b; Zhang *et al.*, 2006). The other site interacts with calmodulin and with the addition of Ca^{2+} , can result in its activation, resulting in translocation to the nucleus and phosphorylation of CREB (Deisseroth *et al.*, 2003).

Somatodendritic release of VP and OT regulates the efficacy of synapses onto the MNCs and somatodendritic release of VP, OT, and other peptides (including dynorphin) regulate MNC firing patterns (Ludwig & Leng, 1997; Ludwig, 1998a; Sladek, 2004). It is known that dynorphin receptors undergo translocation to the cell membrane in dehydrated rats (Shuster *et al.*, 1999) and also in hippocampal neurons, L-type Ca^{2+} channels regulate somatodendritic release of dynorphin (Simmons *et al.*, 1995). Thus the increase in L-type Ca^{2+} channels seen during chronic dehydration may be part of a functional adaptation which leads to enhanced release of peptides such as dynorphin to regulate MNC electrical activity along with increasing release of neuropeptides OT

and VP. In addition, blockers of L-type Ca^{2+} channels reduced depolarization-induced somatodendritic OT release (Tobin *et al.*, 2011), and thus L-type Ca^{2+} channels may have a role in somatodendritic neuropeptide release. Also, the phasic burst firing patterns are important for the sustained release of MNC neurohypophysial hormones (Poulain & Wakerley, 1982). It is well known that the Ca^{2+} is a regulator of the depolarizing (DAP) and hyperpolarizing (HAP, AHP) after potentials and influx through of Ca^{2+} through L-type Ca^{2+} channels may have a role in increasing their currents (Andrew & Dudek, 1983; Armstrong *et al.*, 1994). The increase in $\text{Ca}_v1.2$ seen in our *in vitro* model suggests that this may be important in gene upregulation and enhanced hormonal synthesis and release during conditions of hyperosmotic stress.

5.4 Future directions

The significance of osmotically-induced hypertrophy remains unknown. We suggest that hypertrophy involves insertion of membranes to the cell surface that may include membrane-associated proteins and channels. These may be Ca^{2+} channels (Zhang *et al.*, 2007a), mechanosensitive cation channels (Morenilla-Palao *et al.*, 2004) or even Na^+ channels (Tanaka *et al.*, 1999). Thus the insertion of these channels may modulate the electrical activity of MNCs, which could be important in the long-term release of VP. Subsequent investigations could include patch clamp electrophysiology to understand whether chronic exposure to hypertonic solutions can modulate the electrophysiology of MNCs by changes in the density of ion channels and proteins. Immunocytochemical techniques can be employed to quantify changes in abundance of surface level proteins and channels after exposure to hypertonic solution.

In addition, it will be important to understand the mechanism of osmotically-induced hypertrophy, specifically by investigating the source of the membrane and the mechanisms that mediate its

insertion. Live cell fluorescence of MNCs with organelle specific dyes can be used to visualize where the membrane comes from. Notable dyes include Hoechst 33342 and ER-Tracker-Red for staining and visualizing the dynamic movement of nuclear and ER membranes, respectively. In addition, it can be hypothesized that the membranes come from vesicles containing channels and receptors from the Golgi apparatus and thus blocking protein trafficking from the Golgi using Brefeldin A might block osmotically-evoked hypertrophy.

As described, in addition to insertion of new membrane, an increase in cell volume must accompany an increase in intracellular osmolality. Further studies should investigate the mechanisms involved, which serve to increase the intracellular osmotic strength. This may include using blockers of ionic and organic molecule transporters to see if hypertrophy is affected. In addition, if hypertrophy results in translocation of transporters to the membrane then immunocytochemical studies can be used to document which transporters are involved.

The transient response seen during recovery with and without dynasore should be further probed to understand why the cell undergoes a rapid and overshooting decrease in cell volume followed by a gradual return to the control size. These studies should investigate the kinetics of endocytosis and also the rapidity of ion and water efflux.

Our data on the mechanisms involved in hypertrophy *in vitro* will help us to develop tools to test whether similar mechanisms underlie hypertrophy *in vivo*. In order to effectively translate *in vitro* findings into more functionally applicable data, an *in situ* model of the SON should be used. Specifically, stereotaxic procedures can be used to perform bilateral infusions of various drugs

(action potential blockers and exocytotic fusion blockers) into one SON and a control vehicle in the contralateral SON. Subsequently, structural changes of the SON after a hyperosmotic challenge can be observed or the overall regulation of fluid balance (plasma VP levels and osmolality) can be measured.

Finally, the observation that acutely isolated MNCs hypertrophy under chronic hypotonic conditions as *in vivo* (Zhang *et al.*, 2001), warrants further investigation into the mechanism of the response. It may be that the decrease in size is due to dynamin-dependent endocytosis while the recovery involves exocytosis. Thus these mechanisms can be further probed by using dynasore and TAT-NSF700 to see whether the hypertrophy response or recovery can be blocked by their presence, respectively.

The increase in L-type Ca^{2+} channel subunit $\text{Cav}1.2$ is an important finding suggesting the role of Ca^{2+} and L-type Ca^{2+} channels in the long-term adaptations of MNCs in chronic hypertonic stress. In hypertrophy, the enlargement of MNCs is dependent on activation of SICs and Ca^{2+} influx (Shah *et al.*, 2014). Further studies should investigate whether blocking the SICs (SB366791) and chelating intracellular Ca^{2+} (BAPTA-AM) will prevent the increase in $\text{Cav}1.2$ expression. Further, it was not possible to discern the abundance of $\text{Cav}1.2$ between the MNC plasma membrane and cytoplasm due to the diffuse staining. Further experiments to investigate translocation can include fluorescently labeled dihydropyridines to visualize the location of the channels (Knaus *et al.*, 1992). Finally the increase in $\text{Cav}1.2$ subunit seen in our studies is not paralleled with an increase in mRNA of L-type Ca^{2+} channels and this finding can argue for an increase in protein synthesis (Zhang *et al.*, 2007a). The same experiments described in this study can be performed in the

presence of blockers of protein synthesis such as rapamycin and cyclohexamide. These subsequent studies will help to elucidate the mechanisms underlying this interesting example of neuroendocrine adaptation and help to understand the regulation of body fluid balance during chronic challenges.

CHAPTER 6: CONCLUSION

The MNCs of the SON have been known to undergo a remarkable soma hypertrophy, when exposed to chronic hyperosmotic stress *in vivo*. These *in vivo* findings are confirmed in an *in vitro* model showing that MNCs initially shrink, but undergo a reversible hypertrophy over the course of 90 minutes. Further, it has been shown that hypertrophy is dose-dependent, independent of cell volume regulation, dependent upon activation of PLC and PKC, can be induced by depolarization and increasing $[Ca^{2+}]_i$, and that recovery from hypertrophy depends upon dynamin-mediated endocytosis. In addition, it has been seen that under chronic hypotonic conditions, MNCs initially enlarge followed by a steady and sustained decrease in MNC soma size. Finally, it has been shown that MNCs exposed to hypertonic conditions for two hours, have increased expression of the L-type Ca^{2+} channel $Ca_v1.2$ subunit, similar to an increase in L-type Ca^{2+} current density in MNCs from overnight dehydrated rats. This thesis has documented the effects of chronic hypertonicity on MNC structure and abundance of the L-type Ca^{2+} channel $Ca_v1.2$ subunit. These findings will help us to understand the mechanisms of the important structural and functional neuroendocrine adaptations used to regulate body fluid balance.

CHAPTER 7: REFERENCES

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